PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07K 14/54 — A2

(11) International Publication Number: **WO 00/23471**

(43) International Publication Date: 27 April 2000 (27.04.00)

(21) International Application Number: PCT/EP99/07800

(22) International Filing Date: 6 October 1999 (06.10.99)

(30) Priority Data:
98203529.7 20 October 1998 (20.10.98) EP

(71) Applicant *(for all designated States except US)*: VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW [BE/BE]; Rijvisschestraat 120, B–9052 Zwijnaarde (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: STEIDLER, Lothar [BE/BE]; Bokslaarstraat 41, B–9160 Lokeren (BE). REMAUT, Erik, Rene [BE/BE]; Bergstraat 7, B–9921 Vinderhoute (BE). FIERS, Walter [BE/BE]; Beukendreef 3, B–9070 Destelbergen (BE).

(74) Common Representative: VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW; Rijvisschestraat 120, B–9052 Zwijnaarde (BE).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: USE OF A CYTOKINE–PRODUCING *LACTOCOCCUS* STRAIN TO TREAT COLITIS

(57) Abstract

The current invention relates to an administraton strategy for the delivery at the intestinal mucosa of cytokines or cytokine antagonists, preferably of acid sensitive anti–inflammatory agents, such as IL10 and/or soluble TNF receptor via the oral route. The prefered feature according to the invention is the inoculation with a suspension of recombinant *Lactococcus lactis* cells, which had been engineered to produce the respective proteins.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Use of a cytokine-producing *Lactococcus* strain to treat colitis.

Summary of the invention

The invention generally relates to an administraton strategy for the delivery at the intestinal mucosa of cytokines, preferably of acid sensitive anti-inflammatory agents, such as IL10 and/or a soluble TNF receptor via the oral route. The prefered feature according to the invention is the inoculation with a suspension of live recombinant *Lactococcus lactis* cells, which had been engineered to produce the respective proteins. As example, mice were used in which a chronic inflammation of the distal colon had been induced by administration with dextran sulfate sodium (DSS). The treatment as scored by histological evaluation clearly resulted in a regression of the inflammation and disease symptoms. The finding is highly unexpected since, in order to exert activity at the colon following oral administration, the delivery system needs to pass the acidic environment of the stomach and the upper part of the small intestine respectively.

Background to the invention

The immune system in a mammal is diverse and complex and includes natural and adaptive immune mechanisms and reactions. The immune system is often described in terms of either humoral or cellular immune responses. Humoral immunity refers broadly to antibody production and actions by B-cells; cellular immunity is mediated by cells including T-cells, dendritic cells, neutrophiles, monocytes and macrophages. T-cells and B-cells are two categories of lymphocytes.

One of the mechanisms by which the immune system normally acts and regulates itself includes the production of so-called cytokines. It is known that cytokines mediate several positive and negative immune responses. Cytokines normally act by binding to a receptor on a target cell. The activity of cytokines

can be interfered with in several ways, for example by administration of soluble receptors (extracellular domains of the receptor) or by cytokine analogues or derivatives.

IL-10 is a cytokine capable of mediating a number of actions or effects. It is known that IL-10 is involved in controlling the immune responses of different classes or subsets of Th cells (T-helper cells).

Inflammatory bowel disease (IBD) refers to a group of gastrointestinal disorders characterized by a chronic non-specific inflammation of portions of the gastrointestinal tract. Ulcerative colitis (UC) and Crohn's Disease (CD) are the most prominent examples of IBD in humans. They are associated with many symptoms and complications, including growth retardation in children, rectal prolapse, blood in stools (e.g., melena and/or hematochezia), wasting, iron deficiency, and anemia, e.g. iron deficiency anemia and anemia of chronic disease or of chronic inflammation. The etiology or etiologies of IBD are unclear. Reference hereto is made in Wyngaarden and Smith (eds.) *Cecil's Textbook of Medicine* (W.B. Saunders Co. 1985), Berkow (ed.) *The Merck Manual of Diagnosis and Therapy* (Merck Sharp & Dohme Research Laboratories, 1982), and *Harrison's Principles of Internal Medicine*, 12th Ed., McGraw-Hill, Inc. (1991).

The incidence of IBD varies greatly with geographic location. A collaborative study over Europe shows an incidence per 100 000 of 10,4 for UC and of 5,6 for CD with 40% respectively 80% higher incidence for UC and CD in northern centres when compared to those in the south. As both UC and CD are long time affections, they correspond to real disturbances in the quality of life. Crohn's disease has a bimodal age distribution of onset showing striking peaks in incidence at 20 and 50 years of age. A higher incidence and more grievous disease profile is associated with the peak at younger age. This makes CD especially poignant as afflicted adolescents and young adults are virtually deprived form the high expectations form life, so particularly associated with this social group.

Ulcerative colitis refers to a chronic, non-specific, inflammatory, and ulcerative disease having manifestations primarily in the colonic mucosa. It is frequently

characterized by bloody diarrhea, abdominal cramps, blood and mucus in the stools, malaise, fever, anemia, anorexia, weight loss, leukocytosis, hypoalbuminemia, and an elevated erythrocyte sedimentation rate (ESR).

Complications can include hemorrhage, toxic colitis, toxic megacolon, occasional rectovaginal fistulas, and an increased risk for the development of colon cancer.

Ulcerative colitis is also associated with complications distant from the colon, such as arthritis, ankylosing spondylitis, sacroileitis, posterior uveitis, erythema nodosum, pyoderma gangrenosum, and episcleritis.

Treatment varies considerably with the severity and duration of the disease. For instance, fluid therapy to prevent dehydration and electrolyte imbalance is frequently indicated in a severe attack. Additionally, special dietary measures are sometimes useful. Medications include various corticosteroids, sulphasalazine and some of its derivatives, and possibly immunosuppressive drugs.

Crohn's Disease shares many features in common with ulcerative colitis. Crohn's Disease is distinguishable in that lesions tend to be sharply demarcated from adjacent normal bowel, in contrast to the lesions of ulcerative colitis which are fairly diffuse. Additionally, Crohn's Disease predominately afflicts the ileum (ileitis) and the ileum and colon (ileocolitis). In some cases, the colon alone is diseased (granulomatous colitis) and sometimes the entire small bowel is involved (jejunoileitis). In rare cases, the stomach, duodenum, or esophagus are involved. Lesions include a sarcoid-type epithelioid granuloma in roughly half of the clinical cases. Lesions of Crohn's Disease can be transmural including deep ulceration, edema, and fibrosis, which can lead to obstruction and fistula formation as well as abcess formation. This contrasts with ulcerative colitis which usually yields much shallower lesions, although occasionally the complications of fibrosis, obstruction, fistula formation, and abcesses are seen in ulcerative colitis as well.

Treatment is similar for both diseases and includes steroids, sulphasalazine and its derivatives, and immunosuppressive drugs such as cyclosporin A,

mercaptopurine and azathioprine. More recently developed treatments, some still in clinical trials, involve systemic administration (by injection) of TNF blocking compounds such as TNF-antibodies or soluble TNF receptor.

IBD represents a genuine problem in public health because of the absence of etiologic treatment. Although many patients are managed successfully with conventional medical therapy, such as anti-inflammatory corticosteroid treatment, most will have recurrent activity of disease and two-thirds will require surgery.

The cause of inflammatory bowel diseases is unknown. The pathogenesis of CD and UC probably involves interaction between genetic and environmental factors, such as bacterial agents, although no definite etiological agent has been identified so far. The main theory is that abnormal immune response, possibly driven by intestinal microflora, occurs in IBD. However, what is well established is that T-cells play an important role in the pathogenesis. Activated T-cells can produce both anti-inflammatory and pro-inflammatory cytokines. With this knowledge in hand, IBD can be counteracted in a rational manner. Novel anti-inflammatory therapies, which make use of neutralising monoclonal antibodies or anti-inflammatory cytokines, show the possibility to modulate the immune disregulations causative to IBD. A highly prominent and effective new therapy is systemic treatment with anti-TNF monoclonal antibodies as mentioned above. Single intravenous doses, ranging from 5 to 20 $mg.kg^{-1}$, of the cA2 infliximab antibody resulted in a drastic clinical improvement in active Crohn's disease. The use of systemically administered recombinant IL-10 in a 7 day by day treatment regime using doses ranging from 0.5 to 25 $\mu g.kg^{-1}$ showed reduced Crohn's disease activity scores and increased remission. A number of very promising therapies, either tangling pro-inflammatory cytokines or the establishment of T cell infiltrates, are currently emerging from experimental models. All these strategies however require systemic administration. The severe complications of IBD can be seriouly debilitating, and eventually may lead to death.

Detailed description of the invention

In US Patent 5,368,854, assigned to Schering Corp., a method is disclosed using IL-10 to treat inflammatory bowel diseases in mammals. In this method the cytokine is administered to a mammal having an IBD (inflammatory bowel disease). The administration of IL-10 as described in this reference is parenteral such as intravascular and preferably intravenous.

It is obvious however that such a route of administration for a (human) patient suffering from an IBD is not without drawbacks. A much easier and more convenient way is an oral administration of a medicament comprising a cytokine such as IL-10 or a cytokine-antagonist which has a similar therapeutic activity. More importantly, localized release of the therapeutic agent allows for higher efficacy and less unwanted side effects due to systemic activities.

In WO 97/14806, assigned to Cambridge University Technical Services Ltd., a method is disclosed for delivering biologically active polypeptides and/or antigens by using non-invasive bacteria, such as *Lactococcus*, by intranasal administration of said polypeptides in the body, especially at the mucosa.

However to treat an inflammatory bowel disease such as chronic colitis or Crohn's disease with a cytokine like IL-10, which is acid sensitive, is a very delicate and difficult task to accomplish. Therefore a system needs to be developed wherein the active compound (e.g. a cytokine or a soluble receptor) is delivered directly at the place where the compound is expected to exert its activity taken into account the problem of acid sensitivity of many cytokines, in particular of IL-10, and the requirement that after oral administration the delivery vehicle needs to pass the acidic environment of the stomach. Furthermore, various digestive enzymes degrade polypeptides as they pass through the stomach and the gut. Last but not least in-situ administration of the agent may allow to reach therapeutically effective concentrations which are difficult to achieve by more systemic routes of administration because of systemic toxicity or other limitations.

In order to achieve the recovery of a patient suffering from an IBD, it is necessary to restore the damaged cells and the organ comprising said damaged cells, for instance the colon.

The solution to the above described technical problem is achieved by providing the embodiments characterised in the claims.

It is our invention to use a cytokine-producing Gram-positive bacterial strain or a cytokine antagonist producing Gram-positive bacterial strain for the preparation of a medicament to treat inflammatory bowel disease.
Said cytokine or cytokine antagonist to be produced by the bacterial host strain is, for instance, IL-10, a soluble TNF receptor or a cytokine analogue such as the IL-12 derived p40 homodimer (an IL-12 antagonist), an Interferon-γ-antagonist, an IL-1 antagonist or a virus-coded cytokine analogue such as EBV BCRF1 (Baer et al., 1984), whereas the Gram-positive bacterial strain preferably is a *Lactococcus species* and more preferably a *Lactococcus lactis*.
Other Gram-positive bacterial strains to be used for the purpose of the current invention are *Bacillus subtilis*, *Streptococcus gordonii, Staphylococcus xylosus*, or a *Lactobacillus spec.* such as *Lactobacillus bulgaricus*, *Lactobacillus salivarius, Lactobacillus caseï, Lactobacillus helveticus, Lactobacillus delbrueckii* or *Lactobacillus plantarum.*
The inflammatory bowel diseases such as a chronic colitis, Crohn's disease or an ulcerative colitis can be treated according to the invention with an appropriate dosage of the active cytokine compound, preferably IL-10 or soluble TNF receptor, and provides unexpectedly a restoration of the diseased colon to an apparently normal and healthy state.

IL-10 can be administered alone or in combination with at least one additional therapeutic agent. Examples of such agents include corticosteroids, sulphasalazine, derivatives of sulphasalazine, immunosuppresive drugs such as cyclosporin A, mercaptopurine, azathioprine, and another cytokine. The co-administration can be sequential or simultaneous. Co-administration generally means that the multiple (two or more) therapeutics are present in the recipient during a specified time interval. Typically, if a second agent is administered within the half-life of the first agent, the two agents are considered co-

administered.

The invention disclosed herein thus concerns a localised delivery of IL-10 through in situ synthesis by recombinant *L. Lactis*. As a result thereof the inflammation is reduced by 50% in chronic colitis induced with DSS and prevents the onset of colitis in IL-10 -/- 129 Sv/Ev mice. So the method is equally efficient in comparison to powerful, well-established and accepted therapies relying on the systemic administration of anti-inflammatory proteins.

The vector used here, *L. lactis*, is a Gram positive food grade organism which is totally harmless. It is a non-colonising micro-organism. Accurate dosage and timing during treatment, shown here to be of great importance, can thus easily be obtained.

The critical requirement for viability of the vector is shown in the current invention. This indicates the need for in situ synthesis of IL-10. The vector is indeed capable to achieve this by showing de novo synthesis of IL-10 in the colon.

An efficient novel concept for protein based treatment in the intestinal tract is herewith disclosed. The treatment can be given by the oral route, which is farmost desirable for pharmacological formulations. It can exert effects up to the distal colon using a compound with intrinsic sensitivity for the route used. This method bypasses the need for systemic administration. It opens the possibility for the localised delivery of substances, which are unstable or difficult to produce in high quantities. It is intrinsically very cost effective.

This method may answer the question for sustained and localised presence of IL-10 in therapy at concentrations higher than desirable or even achievable through systemic delivery, with regard to latent side effects.

Some terms used in the current description are, for sake of clarity, explained hereafter.

Generally, the term "symptoms" refers to any subjective evidence of disease or of a patient's condition. This includes evidence as perceived by the patient. Examples of symptoms of IBD include diarrhea, abdominal pain, fever, melena, hematochezia, and weight loss.

The term "signs" refers generally to any objective evidence of a disease or condition, usually as perceived by an examining physician or features which would reveal themselves on a laboratory evaluation or other tests such as an ultrasonic study or a radiographic test. Some examples of signs of IBD include abdominal mass, glossitis, aphtous ulcer, anal fissure, perianal fistula, anemia, malabsorption, and iron deficiency. Occasionally, signs and symptoms overlap. For example, the patient complains of blood stools (a symptom), and a laboratory test of a stool sample is positive for blood (a sign).

The phrase "appropriate dosage" or "effective amount" means an amount or dosage sufficient to ameliorate a symptom or sign of an autoimmune condition or of an undesirable or inappropriate inflammatory or immune response. An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the method route and dose of administration and the severity of the side affects.

With "cytokine" is meant a polypeptide factor produced transiently by a range of cell types, acting usually locally, and activating the expression of specific genes by binding to cell surface receptors.

With "antagonist" is meant a compound that binds to but does not activate receptors, hence does inhibit the action of an agonist competitively.
"Agonists" are compounds that bind to and activate receptors (e.g., endogenous ligands such as hormones and neurotransmitters, chemically synthesized compounds, natural products like alkaloids).

Detailed description of the methods used in the current invention.

Culture media

GM17 is M17 (Difco, St. Louis) supplemented with 0.5 w/v % of glucose. GM17E is GM17 supplemented with 5µg/ml of erythromycin. BM9 contains per liter 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 2 mmol of $MgSO_4$, 25 mmol of $NaHCO_3$, 25 mmol of Na_2CO_3, 0.1 mmol of CaCl2, 5 g of glucose and 5 g of casitone (Difco). BM9E is BM9 supplemented with 5µg/ml of erythromycin.

Recombinant DNA techniques.

PCR amplification of DNA was performed with VENT polymerase and using conditions recommended by the manufacturer. DNA modifying enzymes and restriction endonucleases were used under standard conditions and in the buffers recommended by the manufacturers. General molecular cloning techniques and the electrophoresis of DNA and proteins were carried out essentially as described (Sambrook et al., 1990). *L. lactis* was transformed by electroporation of cells grown in the presence of glycine (Wells et al., 1993).

Construction of the expression plasmids.

The plasmid pT1MIL10 (figure 1) was constructed by subcloning a PCR fragment, obtained with the primers (CAGTACAGCCGGGAAGACAAT and GCACTAGTTAGCTTTTCATTTTGAT) and performed on a cDNA clone containing mIL10 coding sequence. For the design of this strategy we made use of the mIL10 cDNA sequence as given in EMBL acc. nr. M37897. By utilization of the above mentioned primers, the mIL10 fragment could be subcloned as a blunt – SpeI fragment, after treatment with kinase and SpeI, in the NaeI-SpeI opened plasmid pT1NX (figure 1), which is a pTREX1 derivative (Wells and Schofield in : Lactic Acid Bacteria: current advances in metabolism, genetics and applications. F. Bozoglu & R. Bibek, Eds., Nato ASI Series H, Vol.98, p. 37. Springer-Verlag, 1996.)

The plasmid pT1TR5AH (figure 1) was constructed by subcloning a PCR

fragment, obtained with the primers (CTGGTCCCTTCTCTTGGTGAC and CCACTAGTCTATTAATGATGATGATGATGATGCGCAGTACCTGAGTCCTGG GG) and performed on a cDNA clone containing sTNFr55 coding sequence. For the design of this strategy we made use of the TNFr55 cDNA sequence as given in EMBL acc. nr. L26349. By utilizing the above mentioned primers, the sTNFr 55 fragment was provided with a 6his tag at the 3'end and could be subcloned as a blunt – SpeI fragment, after treatment with kinase and SpeI, in the NaeI-SpeI opened plasmid pT1NX.

Both plasmids code, downstream from the lactococcal P1 promotor, for fusion genes between the secretion leader from Usp45 (Van Asseldonk et al., Gene, 95, 155-160,1990) and mIL10 and sTNFr 55, respectively. Upon secretion, the leader sequence is cleaved off.

Identification of recombinant proteins

Recombinant mIL10 and msTNFr 55 could be observed in the supernatant of cultures of MG1363[pT1MIL10] and MG1363[pT1TR5AH], respectively (figure 2). For this test, 5 ml aliquots of the cultures were extracted with 2 ml phenol and the proteins were subsequently prepared from the organic phase by precipitation with 10 ml of ethanol. A part of the precipitate, equivalent to 1 ml of culture supernatant, was subjected to SDS-15% PAGE and immunoblotting. Culture samples were taken at relevant times in the growth phase of the bacteria, as described below.

The culture supernatant of MG1363[pT1MIL10] contained, on average, 1 $\mu g.ml^{-1}$ of murine IL10. Murine IL-10 activity of the supernatant was measured using a murine mast cell line MC/9 (Thompson-Snipes, L. et al., J. Exp. Med. 173, 507, 1991). Human IL-10 binds to murine IL-10R as was demonstrated by transfection experiments (Ho, A.S.Y et al., PNAS 90, 11267, 1993; Liu, Y. et al., J.Immunol. 152, 1821, 1994). 1 $U.ml^{-1}$ of IL-10 is defined as the amount of IL-10 that is able to inhibit 50% the level of IFN-gamma production of conA activated splenocytes (Fiorentino, D.F. et al., J.Exp.Med. 170, 2081, 1989). The ED50 for this effect is typically 0.3-0.6 $ng.ml^{-1}$. When measured along with a standard of known activity (Biosource International, CA) the MG1363[pT1MIL10] culture

supernatant revealed an activity of approximately 8000 $U.ml^{-1}$. Berg et al. (J. Clin. Invest 98, 1010-1020) report a specific activity of approximately 1.0×10^7 $U.mg^{-1}$ for recombinant mIL10. From these considerations and taking into account the variations in the method used, we concluded that the recombinant mIL10, present in the MG1363[pT1MIL10] culture supernatant, displayed full biological activity. No IL10 activity could be detected in the supernatant of the control cultures, MG1363 or MG1363[pTREX1].

The culture supernatant of strain MG1363[pT1TR5AH] contained, on average, 200 $ng.ml^{-1}$ msTNFr 55. Loetscher et al. (1991) showed that complete inhibition of TNF cytotoxic activity by sTNFr 55 was only obtained from a molar ratio of 1000 : 1 of sTNFr 55 to TNF and higher. The soluble recombinant TNFr 55 which had been recovered from the culture supernatant of MG1363(pT1TR5AH) showed an equal inhibitory effect on TNF as had been reported for the indigenous product. This was demonstrated by mixing up and thus competing out a titration series of TNF with a titration series of recombinant sTNFr and measuring TNF activity in a cytotoxicity assay as described (Espevik, T and Nissen-Meyer, 1986).

Pretreatment of the mice

For the induction of chronic colitis, mice were pre-treated as described by Kojouharoff et al. Clin Exp Immunol 107, 353, 1997. Six to eight weeks old female Balb/c mice received four cycles of treatment with DSS. Each cycle consisted of 5% DSS in the drinking water for 7 days, followed by a 10-day interval during which they received normal drinking water. Four to six weeks after completion of the last DSS cycle, mice were treated with the *L. lactis* strains as indicated.

Legends to the figures.

Figure 1 : Overview of the plasmids used

Figure 1 a : schematic maps of the plasmids used. P1 is the lactococcal P1 promotor as in Waterfield et al, (1995), usp45S is a DNA fragment encoding the secretion signal peptide from the lactococcal Usp45 (van Asseldonck et al, 1990), mil 10 is a DNA fragment encoding the mature part of murine interleukin 10, tr55 is a DNA fragment encoding the soluble part of type 1 TNF receptor, H6 is a fragment encoding 6 histidine residues, Em^r is the erythromycin selection marker.

Figure 1b : DNA sequences of pTREX1 and pT1NX

Figure 1c : DNA sequences of pTIMIL10 and pT1TR5AH

Figure 2 :

Protein profile following SDS-PAGE of the culture supernatant of the indicated strains after immunoblot, revealed with anti-murine interleukin 10 (panel A) or anti-murine type 1 TNF receptor and anti-6 His (panel B) antisera.

Figure 3 :

Average of colon length of groups of mice in which: a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pTREX1 was orally administered, c) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pT1TR5AH was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis strain* MG1363pT1MIL10 was orally administered.

Figure 4 :

Average of epithelial damage score in the distal colon of groups of mice in which : a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pTREX1 was orally administered, c) chronic colitis had been induced

with DSS and to which subsequently *L. lactis* strain MG1363pT1TR5AH was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pT1MIL10 was orally administered.

Figure 5 :

Average of inflamatory infiltrate score in the distal colon of groups of mice in which : a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pTREX1 was orally administered, c) chronic colitis had been induced with DSS and to which subsequently *L. lactis strain* MG1363pT1TR5AH was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain MG1363pT1MIL10 was orally administered.

Figure 6:

Representative sections of mice distal colon stained with haematoxylin and eosin.

normal tissue : untreated animals

DSS colitis : animals pretreated with DSS to acquire chronic colitis

DSS colitis, MG1363pT1MIL10 treatment : animals pretreated with DSS to acquire chronic colitis to which subsequently *L. lactis* strain MG1363pT1MIL10 was orally administered. DSS colitis, MG1363pTREX1 treatment : animals pretreated with DSS to acquire chronic colitis to which subsequently *L. lactis* strain MG1363pTREX1 was orally administered.

Figure 7:

Statistical evaluation of the histology. The colon sections were randomly numbered and interpreted blind. Scores from individual mice were subsequently decoded and the regrouped numbers were analysed statistically. The DSS colitis panel shows histological sumscores for the distal colon of blank mice and of mice induced with DSS to acquire chronic colitis, either untreated or treated with *L. lactis* cultures. The score is a sum of scores for epithelial damage and lymphoid infiltrate, both ranging between 0 and 4. Groups of mice (n = 10) were

alternatively treated with MG1363, MG1363(pTREX1) or MG1363(pT1MIL10) (= IL-10) for two (= 2w) or four (= 4w) weeks. Some of the cultures were irradiated with uv (= + uv) prior to inoculation, which reduced cell viability over 10^6 times. The IL-10-/- colitis panel shows histological sumscores of groups (n = 5) of 7 week old untreated, TREX treated and IL-10 treated female 129 Sv/Ev IL-10-/- mice. The histological score is a sum of the degree of inflammation in the proximal, middle and distal colon, all ranging between 0 and 4. Error bars represent s.e.m.

Figure 8:
Representation of bacterial viability after irradiation as measured at OD_{600}.

In order to further disclose and thus clarify the current invention some examples are given hereunder.

Examples

Example 1.

Treatment of the mice with live *L. lactis*

Storage of expression strains.
Freshly streaked cultures of the *L. lactis* expression strains were inoculated in 10 ml of GM17 or GM17E depending on the absence or presence of an expression plasmid and grown overnight at 30°C. The overnight cultures were diluted 1/100 in fresh GM17 or GM17E and pregrown for 3 hours at 30°C. The cells were harvested by centrifugation and resuspended in BGM9 or BGM9E, depending on the presence of plasmids. These cultures were grown for 5 hours at 30°C. The protein profile of these cultures was analysed by performing Western immunoblotting on an equivalent of 1 ml of culture supernatant using either antiserum directed towards sTNFr 55 or IL10 respectively. The protein profile showed the presence of sTNFr 55 and IL10 in the appropriate lanes

(figure 2). 5 ml of the original GM17 or GM17E overnight cultures was supplemented with 5 ml of glycerol and stored at –20°C. These stocks were used as starter material for several experiments. Protein analysis throughout a series of individual experiments showed that a high degree of reproducibility in the production of the recombinant proteins could be obtained by this procedure.

Weeks 1 and 2

Stock solutions of *L. lactis* strains were diluted 1/200 in 10 ml GM17 or GM17E and grown overnight at 30°C. The cells were harvested by centrifugation and resuspended in 1 ml BM9 or BM9E. Control, healthy mice and mice with induced colitis were inoculated on a daily basis with 100 µl aliquots of these cell suspensions.

Weeks 3 and 4

Stock solutions of *L. lactis* strains were diluted 1/200 in 10 ml GM17 or GM17E and grown overnight at 30°C. These cultures were diluted 1/25 in 10 ml of BM9 or BM9E and grown for 3 hours at 30°C. Aliquots of 200 µl were intragastrically (peroral) administered into mice on a daily basis.

Example 2.

Determination of histological score

Histological score was determined essentially as described by Kojouharoff et al. Clin Exp Immunol 107, 353, 1997.

Mice were killed by cervical dislocation. The colon was removed and washed with PBS. The distal third of the colon was cut longitudinally, laid on filter paper and fixed with 10% formalin in PBS overnight. Sections of the parafin-embedded material were made longitudinally. Three 3-µm sections were cut at an intermediate distance of 200 µm. The sections were stained with haematoxylin-eosin. Histological analysis was performed in blind fashion. Mice were scored individually, and each score represented the mean of three sections.

Histology was scored as follows:

Infiltration : 0, no infiltrate; 1, infiltrate around crypt bases; 2, Infiltrate reaching to L. muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant oedema; 4 infiltration of the L. submucosa.

Epithelial damage: 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas and/or foci of polyploid regeneration.

Colonic length was measured immediately after dissection and placement on a paper towel.

The pathology of chronic colitis is, amongst other parameters, characterised by a decrease in length of the colon and by epithelial damage and infiltration of lymphocytes to a more or less substantial extent.

Figure 3 clearly shows an increase in colon length after the treatment of the inflamed mice with MG1363[pT1MIL10] and, although to a lesser extent, after the treatment of the mice with MG1363[pT1TR5AH].

Figure 4 and 5 show the onset of recovery from chronic colitis, in which mice treated with MG1363(pT1MIL10) appear to improve more extensively than those mice which had been treated with MG1363[pT1TR5AH].

Figure 4 shows the histological score of epithelial damage whereas figure 5 shows inflammatory infiltrate, both determined as described previously.

Figure 6 shows the histology of normal tissue, compared to inflamed and treated tissue.

In the normal histology one can observe a continuous array of crypts of equal length. In the crypts, numerous goblet cells can be observed. A low number of lymphocytes is present in the mucosa. No lymphocytes are present in the submucosa. In the inflamed tissue, one can see the disappearance of the organised crypt structures, ranging from differences in length to complete absence of structure. Also, in the relicts of the crypts no goblet cells are present. One can observe a large increase of the thickness of the mucosa due to a massive infiltration of lymphocytes. The lymphocytes tend to form

ulcerations. In severe cases, infiltration of lymphocytes can also be observed in the submucosa. The epithelium, however, remains intact. The negative control of treatment with MG1363(pTREX1) shows a pathology reminescent of that of heavilly inflamed tissue. Mice treated with MG1363 (pT1MIL10) show an almost complete restitution of the normal histology, revealing only slight remainders of infiltrating lymphocytes in the mucosa. Mice treated with MG1363[pT1TR5AH] show an intermediate degree in pathology.

Figure 7 shows the statistic evaluation of histological scores obtained from individual mice following treatment with the indicated *L. lactis* strains (group size = 10). The score was recorded after blind interpretation of slides from the distal colon as described (Kojouharoff et al.,1997). Each mouse was interpreted according to 3 longitudinal slides, equally spaced over the circumference of the colon. Both lymphoid infiltrate and epithelial damage were rated from 0 to 4 points and values for both parameters were summed for every mouse. Normal blank mice showed a histological score of 1 point. The mice induced for colitis are slightly over 5 points. All of the control groups for *L. lactis* treatment fluctuate around this number, with possibly a slightly higher tendency in some groups. The mice treated for 14 days with mIL-10 producing *L. lactis*, followed by 14 days of recovery however show an average of approximately 3 points. This is a decrease of nearly 50% in the pathology when measured against the difference between untreated and blank control groups. The reduction is significant ($p = 0.0151$).

Example 3

Due to the culture conditions used, a minor amount (40 ng) of mIL-10 is present in the supernatant of the inoculation suspension. To investigate whether this IL-10 brings about the observed reduction in the histological score we included treatment with UV killed IL-10 producer strains. These cultures were UV irradiated immediately prior to the inoculation. Figure 8 shows that irradiation reduced the bacterial viability to less than 1 in 10^6 cfu so that no further accumulation of IL-10 was observed. This was not associated with cell lysis

since no drop in OD_{600} was observed and no IL-10 precursor could be detected in the culture supernatant. The irradiation does not affect IL-10 bioactivity. Diseased mice treated for 2 or 4 weeks with the UV dispatched cultures show no difference in colon histology when compared to any of the control groups positive for enterocolitis. The fate of the residual IL-10 in the inoculation medium is most likely denaturation and breakdown in the stomach and duodenum. The acidity of the stomach, prior at pH 1,5, rises to pH6 immediately after inoculation. After 5 minutes a pH of 4 is reached, which further drops from 3,5 to 2,5 in the interval between 30 and 60 minutes after inoculation. IL-10 detected in the stomach 5 minutes after inoculation rapidly decreases in concentration and was only found in trace amounts in the duodenum at 30 minutes after inoculation. At later time-points no IL-10 was detected here nor in the jejunum or ileum.

Example 4

Seven serial inoculations of $3,4.10^9$ cfu of MG1363(pT1MIL10) were given to 129 Sv/Ev IL-10-/- mice, thereby respecting 1 hour intervals. The intestine was prepared out 30 minutes after the last inoculation and divided in the morphologic compartments. Immediately the tissues were homogenised in PBS with 1% BSA and 0,05% NaN_3. Cfu of MG1363(pT1MIL10) were determined as 7.10^6 in the stomach, $2,6.10^8$ in the duodenum, $2,8.10^7$ in the jejunum, 4.10^8 in the ileum, $8,4.10^8$ in the caecum and 7.10^8 in the colon. We have detected 70 ng of soluble IL-10 in the colon homogenate. None of the upstream compartments showed any IL-10 content. From this it is concluded that recombinant *L. lactis* can actively produce IL-10 in the colon.

Example 5

Prevention of enterocolitis in IL10-/- mice

The capacity of the approach described above was tested to prevent the onset of colitis in 129 Sv/Ev IL10-/- mice. These mice spontaneously develop a generalized enterocolitis in the frame between three and eight weeks of age (Kuhn et al.,Cell,1993;75:263-274). Inflammatory changes first appear in the cecum, ascending and transverse colon of 3-wk-old mutants. Progressive disease in ageing IL10-/- mice was characterised by an increased number of multifocal inflammatory cell infiltrates composed of mononuclear cells and neutrophils accompanied by moderate epithelial hyperplasia and slight mucin depletion from goblet cells. Small epithelial erosions and crypt abscesses were occasionally present and inflammation rarely involved the submucosa. IL10-/- mice used in our studies showed a less severe inflammation as described due to "clean" rather than "conventional" conditions of our animal facility.

When these mice are treated from week 3 on for 6 to 8 weeks with either anti IFN-γ or anti-IL-12 colitis can be prevented (Rennick et al., J-Leukoc-Biol.,1997 Apr; 61(4):389-396). We treated 3 weeks old mice by daily intra-gastric inoculation with IL-10 producing *L. lactis*. The mice were treated for 4 weeks with either mid-log or end-log cultures whilst an untreated group was kept under identical conditions. Figure 7 shows histological scores obtained as described (Berg et al., J-Clin-Invest;1996, Aug 15;98(4):1010-1020), with the exception that we did not examine the caecum. The non-treated mice show a mean histological score of approximately 4,5 points. This fits well with reported data, provided one takes into account the contribution of the caecal scores in these values and the slight age difference. The group of mice treated with MG1363(pT1MIL10) shows a mean histological score of 1,5 points which is only slightly over values reported for 3 week old mice (Berg et al., J-Clin-Invest;1996, Aug 15;98(4):1010-1020). As it is the sum of 3 values ranging from 0 to 4 points, this is considered as a very low score. From these data it is clear that the development of colitis can be prevented by this treatment.

References

Wells J.M., & Schofield, K.M. Cloning and expression vectors for lactococci From: Lactic Acid Bacteria (eds Bozoglu B., and Ray, B.) NATO ASI Series H 98: 37-63 Springer-Verlag, Berlin, Heidelberg (1996).

Kojouharoff, G., Hans, W., Obermeier, F., Mannel, D.N., Andus, T., Scholmerich, J., Gross, V. & Falk; W. Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. Clin. Exp. Immunol. 107, 353 - 358, 1997.

Van Asseldonk, M., Rutten, G., Oteman, M., Siezen, R.J., de Vos, W.M. and Simons, G. Cloning of usp45, a gene encoding a secreted protein from *Lactococcus lactis* subsp. *lactis* MG1363. Gene 95, 155-160 (1990).

Sambrook, J., Fritsch, E.F., and Maniatis T. Molecular cloning-a laboratory manual. Cold Spring Harbor Laboratory, New York (1990).

Wells, J.M., Wilson, P.W., and Le Page, R.W.F. Improved cloning vectors and transformation procedure for Lactococcus lactis. J. Appl. Bacteriol. 74, 629-636 (1993).

Schlaak, J.F., Schmitt, E., Huls, C., Meyer zum Buschenfelde, K.H. & Fleischer, B. A sensitive and specific bioassay for the detection of human interleukin-10. J. Immunol. Methods 168, 49-54, 1994.

Thompson-Snipes, L., Dhar, V., Bond, M.W., Mosmann, T.R., Moore, K.W. & Rennick, DM Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. J. Exp. Med. 173, 507-10, 1991.

Ho, A., S., Y., Liu, Y., Khan, T., A., Hsu, D., H., Bazan, J., F. & Moore, K., W. A receptor for interleukin 10 is related to interferon receptors. Proceedings of the National Academy of Sciences of the United States of America 90(23): 11267-11271 (1993)

Liu, Y., Wei, S., H., Y., Ho, A., S., Y., De Waal-Malefyt, R. & Moore, K., W. Expression cloning and characterization of a human IL-10 receptor. Journal of Immunology 152(4): 1821-1829 (1994)

Fiorentino, D.F., Bond, M.W. & Mosmann, T.R. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J-Exp-Med. 170, 2081-95, 1989.

Waterfield,N.R. et al., The isolation of lactococcal promoters and their use in investigating bacterial luciferase synthesis in lactococcus lactis. Gene,165,9-15 (1995).

Baer, R. et al., DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature,130, 207-211 (1984).

Claims

1. Use of a cytokine-producing Gram-positive bacterial strain or a cytokine antagonist-producing Gram-positive bacterial strain for the preparation of a medicament to treat inflammatory bowel disease.
2. Use of a Gram-positive bacterial strain according to claim 1 wherein the cytokine or cytokine antagonist is IL-10, a soluble TNF receptor or another TNF antagonist, an IL-12 antagonist, an Interferon-γ antagonist, an IL-1 antagonist or a virus-coded cytokine analogue such as EBV BCRF1.
3. Use of a Gram-positive bacterial strain according to claim 1 or 2 wherein the Gram-positive bacterial strain is a *Lactococcus species.*
4. Use of a Gram-positive bacterial strain according to claim 3 wherein the *Lactococcus species* is *Lactococcus lactis.*
5. Use of a Gram-positive bacterial strain according to claim 1 or 2 wherein the Gram-positive bacterial strain is *Bacillus subtilis*, *Streptococcus gordonii, Staphylococcus xylosus*, or a *Lactobacillus spec.*
6. Use of a Gram-positive bacterial strain according to any of the preceeding claims wherein the bowel disease is a chronic colitis, Crohn's disease or an ulcerative colitis.

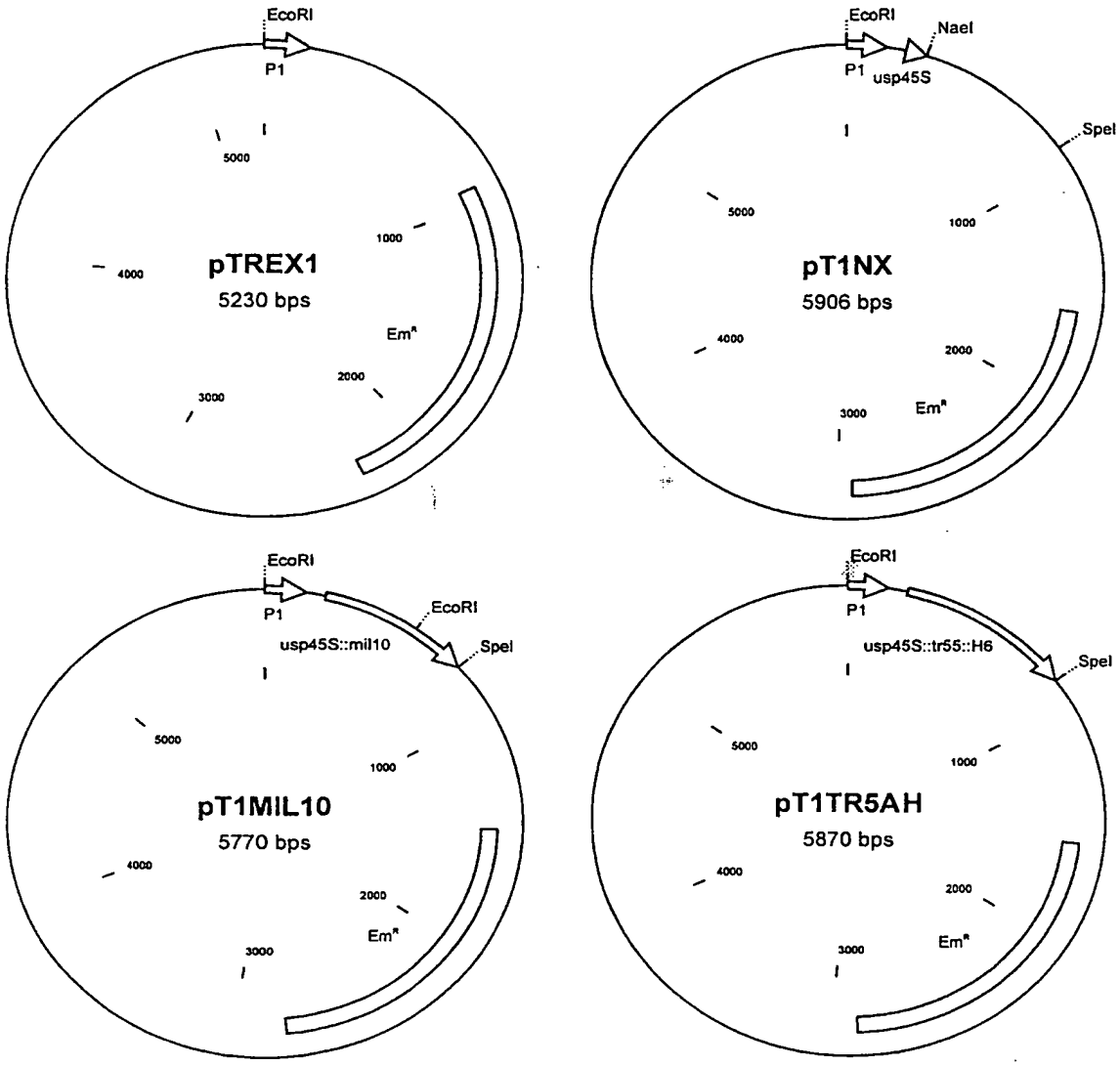
EcoRI
P1
pTREX1
5230 bps
Em^R
EcoRI
NaeI
P1
usp45S
SpeI
pT1NX
5906 bps
Em^R
EcoRI
P1
usp45S::mil10
EcoRI
SpeI
pT1MIL10
5770 bps
Em^R
EcoRI
P1
usp45S::tr55::H6
SpeI
pT1TR5AH
5870 bps
Em^R

Figure 1a

pTREX1 Figure 1B

GAATTCGATTAAGTCATCTTACCTCTTTTATTAGTTTTTTCTTATAATCTAATGATAACATTT
TTATAATTAATCTATAAACCATATCCCTCTTTGGAATCAAAATTTATTATCTACTCCTTTGTA
GATATGTTATAATACAAGTATCAGATCTGGGAGACCACAACGGTTTCCCACTAGAAATAA
TTTTGTTTAACTTTAGAAAGGAGATATACGCATGCAGGATATCTCTAGAATGGATCCGGC
TGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAG
CATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACT
ATATCCGGATGACCTGCAGGCAAGCTCTAGAATCGATACGATTTTGAAGTGGCAACAGA
TAAAAAAAAGCAGTTTAAAATTGTTGCTGAACTTTTAAAACAAGCAAATACAATCATTGTC
GCAACAGATAGCGACAGAGAAGGCGAAAACATTGCCTGGTCGATCATTCATAAAGCAAA
TGCCTTTTCTAAAGATAAAACGTATAAAAGACTATGGATCAATAGTTTAGAAAAAGATGTG
ATCCGTAGCGGTTTTCAAAATTTGCAACCAGGAATGAATTACTATCCCTTTTATCAAGAAG
CGCAAAAGAAAAACGAAATGATACACCAATCAGTGCAAAAAAAGATATAATGGGAGATAA
GACGGTTCGTGTTCGTGCTGACTTGCACCATATCATAAAAATCGAAACAGCAAAGAATGG
CGGAAACGTAAAAGAAGTTATGGAAATAAGACTTAGAAGCAAACTTAAGAGTGTGTTGAT
AGTGCAGTATCTTAAAATTTTGTATAATAGGAATTGAAGTTAAATTAGATGCTAAAAATTTG
TAATTAAGAAGGAGTGATTACATGAACAAAAATATAAAATATTCTCAAAACTTTTTAACGA
GTGAAAAAGTACTCAACCAAATAATAAAACAATTGAATTTAAAAGAAACCGATACCGTTTA
CGAAATTGGAACAGGTAAAGGGCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGG
TAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAA
TACTCGTGTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGG
TATAAAATTGTTGGGAGTATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGTTTT
TGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTT
GGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTGCT
TAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACT
TACCCGCCATACCACAGATGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCA
AAATGGGTCAATCGAGAATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCAATGA
AACACGCCAAAGTAAACAATTTAAGTACCGTTACTTATGAGCAAGTATTGTCTATTTTTAA
TAGTTATCTATTATTTAACGGGAGGAAATAATTCTATGAGTCGCTTTTGTAAATTTGGAAA
GTTACACGTTACTAAAGGGAATGTAGATAAATTATTAGGTATACTACTGACAGCTTCCAA
GGAGCTAAAGAGGTCCCTAGCGCTCTTATCATGGGGAAGCTCGGATCATATGCAAGACA
AAATAAACTCGCAACAGCACTTGGAGAAATGGGACGAATCGAGAAAACCCTCTTTACGC
TGGATTACATATCTAATAAAGCCGTAAGGAGACGGGTTCAAAAAGGTTTAAATAAAGGAG
AAGCAATCAATGCATTAGCTAGAACTATATTTTTTGGACAACGTGGAGAATTTAGAGAAC
GTGCTCTCCAAGACCAGTTACAAAGAGCTAGTGCACTAAACATAATTATTAACGCTATAA
GTGTGTGGAACACTGTATATATGGAAAAAGCCGTAGAAGAATTAAAAGCAAGAGGAGAA
TTTAGAGAAGATTTAATGCCATATGCGTGGCCGTTAGGATGGGAACATATCAATTTTCTT
GGAGAATACAAATTTGAAGGATTACATGACACTGGGCAAATGAATTTACGTCCTTTACGT
ATAAAAGAGCCGTTTTATTCTTAATATAACGGCTCTTTTTATAGAAAAAATCCTTAGCGTG
GTTTTTTTCCGAAATGCTGGCGGTACCCCAAGAATTAGAAATGAGTAGATCAAATTATTC
ACGAATAGAATCAGGAAAATCAGATCCAACCATAAAAACACTAGAACAAATTGCAAAGTT
AACTAACTCAACGCTAGTAGTGGATTTAATCCCAAATGAGCCAACAGAACCAGAGCCAG
AAACAGAATCAGAACAAGTAACATTGGATTTAGAAATGGAAGAAGAAAAAAGCAATGACT
TCGTGTGAATAATGCACGAAATCGTTGCTTATTTTTTTTTAAAAGCGGTATACTAGATATA
ACGAAACAACGAACTGAATAGAAACGAAAAAAGAGCCATGACACATTTATAAAATGTTTG
ACGACATTTTATAAATGCATAGCCCGATAAGATTGCCAAACCAACGCTTATCAGTTAGTC
AGATGAACTCTTCCCTCGTAAGAAGTTATTTAATTAACTTTGTTTGAAGACGGTATATAAC
CGTACTATCATTATATAGGGAAATCAGAGAGTTTTCAAGTATCTAAGCTACTGAATTTAAG
AATTGTTAAGCAATCAATCGGAAATCGTTTGATTGCTTTTTTTGTATTCATTTATAGAAGGT
GGAGTTTGTATGAATCATGATGAATGTAAAACTTATATAAAAAATAGTTTATTGGAGATAA

pTREX1 (cont.) Figure 1B (cont.)

GAAAATTAGCAAATATCTATACACTAGAAACGTTTAAGAAAGAGTTAGAAAAGAGAAATAT
CTACTTAGAAACAAAATCAGATAAGTATTTTTCTTCGGAGGGGGAAGATTATATATATAAG
TTAATAGAAAATAACAAAATAATTTATTCGATTAGTGGAAAAAAATTGACTTATAAAGGAAA
AAAATCTTTTTCAAAACATGCAATATTGAAACAGTTGAATGAAAAAGCAAACCAAGTTAAT
TAAACAACCTATTTTATAGGATTTATAGGAAAGGAGAACAGCTGAATGAATATCCCTTTTG
TTGTAGAAACTGTGCTTCATGACGGCTTGTTAAAGTACAAATTTAAAAATAGTAAAATTCG
CTCAATCACTACCAAGCCAGGTAAAAGCAAAGGGGCTATTTTTGCGTATCGCTCAAAATC
AAGCATGATTGGCGGTCGTGGTGTTGTTCTGACTTCCGAGGAAGCGATTCAAGAAAATC
AAGATACATTTACACATTGGACACCCAACGTTTATCGTTATGGAACGTATGCAGACGAAA
ACCGTTCATACACGAAAGGACATTCTGAAAACAATTTAAGACAAATCAATACCTTCTTTAT
TGATTTTGATATTCACACGGCAAAAGAAACTATTTCAGCAAGCGATATTTTAACAACCGCT
ATTGATTTAGGTTTTATGCCTACTATGATTATCAAATCTGATAAAGGTTATCAAGCATATTT
TGTTTTAGAAACGCCAGTCTATGTGACTTCAAAATCAGAATTTAAATCTGTCAAAGCAGCC
AAAATAATTTCGCAAAATATCCGAGAATATTTTGGAAAGTCTTTGCCAGTTGATCTAACGT
GTAATCATTTTGGTATTGCTCGCATACCAAGAACGGACAATGTAGAATTTTTTGATCCTAA
TTACCGTTATTCTTTCAAAGAATGGCAAGATTGGTCTTTCAAACAAACAGATAATAAGGGC
TTTACTCGTTCAAGTCTAACGGTTTTAAGCGGTACAGAAGGCAAAAAACAAGTAGATGAA
CCCTGGTTTAATCTCTTATTGCACGAAACGAAATTTTCAGGAGAAAAGGGTTTAATAGGG
CGTAATAACGTCATGTTTACCCTCTCTTTAGCCTACTTTAGTTCAGGCTATTCAATCGAAA
CGTGCGAATATAATATGTTTGAGTTTAATAATCGATTAGATCAACCCTTAGAAGAAAAAGA
AGTAATCAAAATTGTTAGAAGTGCCTATTCAGAAAACTATCAAGGGGCTAATAGGGAATA
CATTACCATTCTTTGCAAAGCTTGGGTATCAAGTGATTTAACCAGTAAAGATTTATTTGTC
CGTCAAGGGTGGTTTAAATTCAAGAAAAAAAGAAGCGAACGTCAACGTGTTCATTTGTCA
GAATGGAAAGAAGATTTAATGGCTTATATTAGCGAAAAAAGCGATGTATACAAGCCTTAT
TTAGTGACGACCAAAAAAGAGATTAGAGAAGTGCTAGGCATTCCTGAACGGACATTAGA
TAAATTGCTGAAGGTACTGAAGGCGAATCAGGAAATTTTCTTTAAGATTAAACCAGGAAG
AAATGGTGGCATTCAACTTGCTAGTGTTAAATCATTGTTGCTATCGATCATTAAAGTAAAA
AAAGAAGAAAAAGAAAGCTATATAAAGGCGCTGACAAATTCTTTTGACTTAGAGCATACA
TTCATTCAAGAGACTTTAAACAAGCTAGCAGAACGCCCTAAAACGGACACACAACTCGAT
TTGTTTAGCTATGATACAGGCTGAAAATAAAACCCGCACTATGCCATTACATTTATATCTA
TGATACGTGTTTGTTTTTTCTTTGCTGTTTAGCGAATGATTAGCAGAAATATACAGAGTAA
GATTTTAATTAATTATTAGGGGGAGAAGGAGAGAGTAGCCCGAAAACTTTTAGTTGGCTT
GGACTGAACGAAGTGAGGGAAAGGCTACTAAAACGTCGAGGGGCAGTGAGAGCGAAG
CGAACACTTGATTTTTTAATTTTCTATCTTTTATAGGTCATTAGAGTATACTTATTTGTCCT
ATAAACTATTTAGCAGCATAATAGATTTATTGAATAGGTCATTTAAGTTGAGCATATTAGA
GGAGGAAAATCTTGGAGAAATATTTGAAGAACCCGATTACATGGATTGGATTAGTTCTTG
TGGTTACGTGGTTTTTAACTAAAAGTAGTGAATTTTTGATTTTTGGTGTGTGTGTCTTGTT
GTTAGTATTTGCTAGTCAAAGTGATTAAATA

pT1NX Figure 1B (cont.)

GAATTCGATTAAGTCATCTTACCTCTTTTATTAGTTTTTTCTTATAATCTAATGATAACATTT
TTATAATTAATCTATAAACCATATCCCTCTTTGGAATCAAAATTTATTATCTACTCCTTTGTA
GATATGTTATAATACAAGTATCAGATCTGGGAGACCACAACGGTTTCCCACTAGAAATAA
TTTTGTTTAACTTTAGAAAGGAGATATACGCATGAAAAAAAAGATTATCTCAGCTATTTTAA
TGTCTACAGTCATACTTTCTGCTGCAGCCCCGTTGTCAGGTGTTTACGCCGGCGACGGA
TCCAAAAGAGGAAGACAATAACAAGCCTGGCAAAGAAGACAATAACAAGCCTGGCAAAG
AAGACAATAACAAGCCTGGCAAAGAAGACAACAACAAGCCTGGCAAAGAAGACAACAAC
AAGCCTGGTAAAGAAGACAACAACAAGCCTGGCAAAGAAGACGGCAACAAGCCTGGTAA
AGAAGACAACAAAAAACCTGGTAAAGAAGATGGCAACAAGCCTGGTAAAGAAGACAACA
AAAAACCTGGTAAAGAAGACGGCAACAAGCCTGGCAAAGAAGATGGCAACAAACCTGGT
AAAGAAGATGGTAACGGAGTACATGTCGTTAAACCTGGTGATACAGTAAATGACATTGCA
AAAGCAAACGGCACTACTGCTGACAAAATTGCTGCAGATAACAAATTAGCTGATAAAAAC
ATGATCAAACCTGGTCAAGAACTTGTTGTTGATAAGAAGCAACCAGCAAACCATGCAGAT
GCTAACAAAGCTCAAGCATTACCAGAAACTGGCGAAGAAAATCCATTCATCGGTACAACT
GTATTTGGTGGATTATCATTAGCCTTAGGTGCAGCGTTATTAGCTGGACGTCGTCGCGA
ACTATAACTAGTAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTG
CCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGT
TTTTTGCTGAAAGGAGGAACTATATCCGGATGACCTGCAGGCAAGCTCTAGAATCGATA
CGATTTTGAAGTGGCAACAGATAAAAAAAAGCAGTTTAAAATTGTTGCTGAACTTTTAAAA
CAAGCAAATACAATCATTGTCGCAACAGATAGCGACAGAGAAGGCGAAAACATTGCCTG
GTCGATCATTCATAAAGCAAATGCCTTTTCTAAAGATAAAACGTATAAAAGACTATGGATC
AATAGTTTAGAAAAAGATGTGATCCGTAGCGGTTTTCAAAATTTGCAACCAGGAATGAAT
TACTATCCCTTTTATCAAGAAGCGCAAAAGAAAAACGAAATGATACACCAATCAGTGCAA
AAAAAGATATAATGGGAGATAAGACGGTTCGTGTTCGTGCTGACTTGCACCATATCATAA
AAATCGAAACAGCAAAGAATGGCGGAAACGTAAAAGAAGTTATGGAAATAAGACTTAGAA
GCAAACTTAAGAGTGTGTTGATAGTGCAGTATCTTAAAATTTTGTATAATAGGAATTGAAG
TTAAATTAGATGCTAAAAATTTGTAATTAAGAAGGAGTGATTACATGAACAAAAATATAAA
ATATTCTCAAAACTTTTTAACGAGTGAAAAAGTACTCAACCAAATAATAAAACAATTGAATT
TAAAAGAAACCGATACCGTTTACGAAATTGGAACAGGTAAAGGGCATTTAACGACGAAAC
TGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATC
GTCAGAAAAATTAAAACTGAATACTCGTGTCACTTTAATTCACCAAGATATTCTACAGTTT
CAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAGTATTCCTTACCATTTAAGCACAC
AAATTATTAAAAAAGTGGTTTTTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGA
AGGATTCTACAAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCA
AGTCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGT
AAACAGTGTCTTAATAAAACTTACCCGCCATACCACAGATGTTCCAGATAAATATTGGAA
GCTATATACGTACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACTGTTTACTAAA
AATCAGTTTCATCAAGCAATGAAACACGCCAAAGTAAACAATTTAAGTACCGTTACTTATG
AGCAAGTATTGTCTATTTTTAATAGTTATCTATTATTTAACGGGAGGAAATAATTCTATGAG
TCGCTTTTGTAAATTTGGAAAGTTACACGTTACTAAAGGGAATGTAGATAAATTATTAGGT
ATACTACTGACAGCTTCCAAGGAGCTAAAGAGGTCCCTAGCGCTCTTATCATGGGGAAG
CTCGGATCATATGCAAGACAAAATAAACTCGCAACAGCACTTGGAGAAATGGGACGAAT
CGAGAAAACCCTCTTTACGCTGGATTACATATCTAATAAAGCCGTAAGGAGACGGGTTCA
AAAAGGTTTAAATAAAGGAGAAGCAATCAATGCATTAGCTAGAACTATATTTTTTGGACAA
CGTGGAGAATTTAGAGAACGTGCTCTCCAAGACCAGTTACAAAGAGCTAGTGCACTAAA
CATAATTATTAACGCTATAAGTGTGTGGAACACTGTATATATGGAAAAAGCCGTAGAAGA
ATTAAAAGCAAGAGGAGAATTTAGAGAAGATTTAATGCCATATGCGTGGCCGTTAGGATG
GGAACATATCAATTTTCTTGGAGAATACAAATTTGAAGGATTACATGACACTGGGCAAAT
GAATTTACGTCCTTTACGTATAAAAGAGCCGTTTTATTCTTAATATAACGGCTCTTTTTATA
GAAAAAATCCTTAGCGTGGTTTTTTTCCGAAATGCTGGCGGTACCCCAAGAATTAGAAAT

pT1NX (cont.) Figure 1B (cont.)

GAGTAGATCAAATTATTCACGAATAGAATCAGGAAAATCAGATCCAACCATAAAAACACTA
GAACAAATTGCAAAGTTAACTAACTCAACGCTAGTAGTGGATTTAATCCCAAATGAGCCA
ACAGAACCAGAGCCAGAAACAGAATCAGAACAAGTAACATTGGATTTAGAAATGGAAGA
AGAAAAAAGCAATGACTTCGTGTGAATAATGCACGAAATCGTTGCTTATTTTTTTTTAAAA
GCGGTATACTAGATATAACGAAACAACGAACTGAATAGAAACGAAAAAAGAGCCATGACA
CATTTATAAAATGTTTGACGACATTTTATAAATGCATAGCCCGATAAGATTGCCAAACCAA
CGCTTATCAGTTAGTCAGATGAACTCTTCCCTCGTAAGAAGTTATTTAATTAACTTTGTTT
GAAGACGGTATATAACCGTACTATCATTATATAGGGAAATCAGAGAGTTTTCAAGTATCTA
AGCTACTGAATTTAAGAATTGTTAAGCAATCAATCGGAAATCGTTTGATTGCTTTTTTTGT
ATTCATTTATAGAAGGTGGAGTTTGTATGAATCATGATGAATGTAAAACTTATATAAAAAA
TAGTTTATTGGAGATAAGAAAATTAGCAAATATCTATACACTAGAAACGTTTAAGAAAGAG
TTAGAAAAGAGAAATATCTACTTAGAAACAAAATCAGATAAGTATTTTTCTTCGGAGGGG
GAAGATTATATATATAAGTTAATAGAAAATAACAAAATAATTTATTCGATTAGTGGAAAAAA
ATTGACTTATAAAGGAAAAAAATCTTTTTCAAAACATGCAATATTGAAACAGTTGAATGAA
AAAGCAAACCAAGTTAATTAAACAACCTATTTTATAGGATTTATAGGAAAGGAGAACAGCT
GAATGAATATCCCTTTTGTTGTAGAAACTGTGCTTCATGACGGCTTGTTAAAGTACAAATT
TAAAAATAGTAAAATTCGCTCAATCACTACCAAGCCAGGTAAAAGCAAAGGGGCTATTTT
TGCGTATCGCTCAAAATCAAGCATGATTGGCGGTCGTGGTGTTGTTCTGACTTCCGAGG
AAGCGATTCAAGAAAATCAAGATACATTTACACATTGGACACCCAACGTTTATCGTTATG
GAACGTATGCAGACGAAAACCGTTCATACACGAAAGGACATTCTGAAAACAATTTAAGAC
AAATCAATACCTTCTTTATTGATTTTGATATTCACACGGCAAAAGAAACTATTTCAGCAAG
CGATATTTTAACAACCGCTATTGATTTAGGTTTTATGCCTACTATGATTATCAAATCTGATA
AAGGTTATCAAGCATATTTTGTTTTAGAAACGCCAGTCTATGTGACTTCAAAATCAGAATT
TAAATCTGTCAAAGCAGCCAAAATAATTTCGCAAAATATCCGAGAATATTTTGGAAAGTCT
TTGCCAGTTGATCTAACGTGTAATCATTTTGGTATTGCTCGCATACCAAGAACGGACAAT
GTAGAATTTTTTGATCCTAATTACCGTTATTCTTTCAAAGAATGGCAAGATTGGTCTTTCA
AACAAACAGATAATAAGGGCTTTACTCGTTCAAGTCTAACGGTTTTAAGCGGTACAGAAG
GCAAAAAACAAGTAGATGAACCCTGGTTTAATCTCTTATTGCACGAAACGAAATTTTCAG
GAGAAAAGGGTTTAATAGGGCGTAATAACGTCATGTTTACCCTCTCTTTAGCCTACTTTA
GTTCAGGCTATTCAATCGAAACGTGCGAATATAATATGTTTGAGTTTAATAATCGATTAGA
TCAACCCTTAGAAGAAAAAGAAGTAATCAAAATTGTTAGAAGTGCCTATTCAGAAAACTAT
CAAGGGGCTAATAGGGAATACATTACCATTCTTTGCAAAGCTTGGGTATCAAGTGATTTA
ACCAGTAAAGATTTATTTGTCCGTCAAGGGTGGTTTAAATTCAAGAAAAAAAGAAGCGAA
CGTCAACGTGTTCATTTGTCAGAATGGAAAGAAGATTTAATGGCTTATATTAGCGAAAAA
AGCGATGTATACAAGCCTTATTTAGTGACGACCAAAAAAGAGATTAGAGAAGTGCTAGG
CATTCCTGAACGGACATTAGATAAATTGCTGAAGGTACTGAAGGCGAATCAGGAAATTTT
CTTTAAGATTAAACCAGGAAGAAATGGTGGCATTCAACTTGCTAGTGTTAAATCATTGTTG
CTATCGATCATTAAAGTAAAAAAAGAAGAAAAAGAAAGCTATATAAAGGCGCTGACAAAT
TCTTTTGACTTAGAGCATACATTCATTCAAGAGACTTTAAACAAGCTAGCAGAACGCCCT
AAAACGGACACACAACTCGATTTGTTTAGCTATGATACAGGCTGAAAATAAAACCCGCAC
TATGCCATTACATTTATATCTATGATACGTGTTTGTTTTTTCTTTGCTGTTTAGCGAATGAT
TAGCAGAAATATACAGAGTAAGATTTTAATTAATTATTAGGGGGAGAAGGAGAGAGTAGC
CCGAAAACTTTTAGTTGGCTTGGACTGAACGAAGTGAGGGAAAGGCTACTAAAACGTCG
AGGGGCAGTGAGAGCGAAGCGAACACTTGATTTTTTAATTTTCTATCTTTTATAGGTCATT
AGAGTATACTTATTTGTCCTATAAACTATTTAGCAGCATAATAGATTTATTGAATAGGTCAT
TTAAGTTGAGCATATTAGAGGAGGAAAATCTTGGAGAAATATTTGAAGAACCCGATTACA
TGGATTGGATTAGTTCTTGTGGTTACGTGGTTTTTAACTAAAAGTAGTGAATTTTTGATTT
TTGGTGTGTGTGTCTTGTTGTTAGTATTTGCTAGTCAAAGTGATTAAATA

pT1MIL10 Figure 1c

GAATTCGATTAAGTCATCTTACCTCTTTTATTAGTTTTTTCTTATAATCTAATGATAACATTT
TTATAATTAATCTATAAACCATATCCCTCTTTGGAATCAAAATTTATTATCTACTCCTTTGTA
GATATGTTATAATACAAGTATCAGATCTGGGAGACCACAACGGTTTCCCACTAGAAATAA
TTTTGTTTAACTTTAGAAAGGAGATATACGCATGAAAAAAAAGATTATCTCAGCTATTTTAA
TGTCTACAGTCATACTTTCTGCTGCAGCCCCGTTGTCAGGTGTTTACGCCCAGTACAGC
CGGGAAGACAATAACTGCACCCACTTCCCAGTCGGCCAGAGCCACATGCTCCTAGAGCT
GCGGACTGCCTTCAGCCAGGTGAAGACTTTCTTTCAAACAAAGGACCAGCTGGACAACA
TACTGCTAACCGACTCCTTAATGCAGGACTTTAAGGGTTACTTGGGTTGCCAAGCCTTAT
CGGAAATGATCCAGTTTTACCTGGTAGAAGTGATGCCCCAGGCAGAGAAGCATGGCCCA
GAAATCAAGGAGCATTTGAATTCCCTGGGTGAGAAGCTGAAGACCCTCAGGATGCGGCT
GAGGCGCTGTCATCGATTTCTCCCCTGTGAAAATAAGAGCAAGGCAGTGGAGCAGGTG
AAGAGTGATTTTAATAAGCTCCAAGACCAAGGTGTCTACAAGGCCATGAATGAATTTGAC
ATCTTCATCAACTGCATAGAAGCATACATGATGATCAAAATGAAAAGCTAACTAGTAGATC
CGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA
ACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAG
GAACTATATCCGGATGACCTGCAGGCAAGCTCTAGAATCGATACGATTTTGAAGTGGCA
ACAGATAAAAAAAAGCAGTTTAAAATTGTTGCTGAACTTTTAAAACAAGCAAATACAATCA
TTGTCGCAACAGATAGCGACAGAGAAGGCGAAAACATTGCCTGGTCGATCATTCATAAA
GCAAATGCCTTTTCTAAAGATAAAACGTATAAAAGACTATGGATCAATAGTTTAGAAAAAG
ATGTGATCCGTAGCGGTTTTCAAAATTTGCAACCAGGAATGAATTACTATCCCTTTTATCA
AGAAGCGCAAAAGAAAAACGAAATGATACACCAATCAGTGCAAAAAAAGATATAATGGGA
GATAAGACGGTTCGTGTTCGTGCTGACTTGCACCATATCATAAAAATCGAAACAGCAAAG
AATGGCGGAAACGTAAAAGAAGTTATGGAAATAAGACTTAGAAGCAAACTTAAGAGTGTG
TTGATAGTGCAGTATCTTAAAATTTTGTATAATAGGAATTGAAGTTAAATTAGATGCTAAAA
ATTTGTAATTAAGAAGGAGTGATTACATGAACAAAAATATAAAATATTCTCAAAACTTTTTA
ACGAGTGAAAAAGTACTCAACCAAATAATAAAACAATTGAATTTAAAAGAAACCGATACCG
TTTACGAAATTGGAACAGGTAAAGGGCATTTAACGACGAAACTGGCTAAAATAAGTAAAC
AGGTAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACT
GAATACTCGTGTCACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAG
AGGTATAAAATTGTTGGGAGTATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGG
TTTTTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTA
CCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAAT
TGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAA
AACTTACCCGCCATACCACAGATGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGT
TTCAAAATGGGTCAATCGAGAATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCA
ATGAAACACGCCAAAGTAAACAATTTAAGTACCGTTACTTATGAGCAAGTATTGTCTATTT
TTAATAGTTATCTATTATTTAACGGGAGGAAATAATTCTATGAGTCGCTTTTGTAAATTTG
GAAAGTTACACGTTACTAAAGGGAATGTAGATAAATTATTAGGTATACTACTGACAGCTTC
CAAGGAGCTAAAGAGGTCCCTAGCGCTCTTATCATGGGGAAGCTCGGATCATATGCAAG
ACAAAATAAACTCGCAACAGCACTTGGAGAAATGGGACGAATCGAGAAAACCCTCTTTAC
GCTGGATTACATATCTAATAAAGCCGTAAGGAGACGGGTTCAAAAAGGTTTAAATAAAGG
AGAAGCAATCAATGCATTAGCTAGAACTATATTTTTTGGACAACGTGGAGAATTTAGAGA
ACGTGCTCTCCAAGACCAGTTACAAAGAGCTAGTGCACTAAACATAATTATTAACGCTAT
AAGTGTGTGGAACACTGTATATATGGAAAAAGCCGTAGAAGAATTAAAAGCAAGAGGAG
AATTTAGAGAAGATTTAATGCCATATGCGTGGCCGTTAGGATGGGAACATATCAATTTTC
TTGGAGAATACAAATTTGAAGGATTACATGACACTGGGCAAATGAATTTACGTCCTTTAC
GTATAAAAGAGCCGTTTTATTCTTAATATAACGGCTCTTTTTATAGAAAAAATCCTTAGCG
TGGTTTTTTTCCGAAATGCTGGCGGTACCCCAAGAATTAGAAATGAGTAGATCAAATTAT
TCACGAATAGAATCAGGAAAATCAGATCCAACCATAAAAACACTAGAACAAATTGCAAAG

pT1MIL10 (cont.) Figure 1c (cont.)

TTAACTAACTCAACGCTAGTAGTGGATTTAATCCCAAATGAGCCAACAGAACCAGAGCCA
GAAACAGAATCAGAACAAGTAACATTGGATTTAGAAATGGAAGAAGAAAAAAGCAATGAC
TTCGTGTGAATAATGCACGAAATCGTTGCTTATTTTTTTTTAAAAGCGGTATACTAGATAT
AACGAAACAACGAACTGAATAGAAACGAAAAAAGAGCCATGACACATTTATAAAATGTTT
GACGACATTTTATAAATGCATAGCCCGATAAGATTGCCAAACCAACGCTTATCAGTTAGT
CAGATGAACTCTTCCCTCGTAAGAAGTTATTTAATTAACTTTGTTTGAAGACGGTATATAA
CCGTACTATCATTATATAGGGAAATCAGAGAGTTTTCAAGTATCTAAGCTACTGAATTTAA
GAATTGTTAAGCAATCAATCGGAAATCGTTTGATTGCTTTTTTTGTATTCATTTATAGAAG
GTGGAGTTTGTATGAATCATGATGAATGTAAAACTTATATAAAAAATAGTTTATTGGAGAT
AAGAAAATTAGCAAATATCTATACACTAGAAACGTTTAAGAAAGAGTTAGAAAAGAGAAAT
ATCTACTTAGAAACAAAATCAGATAAGTATTTTTCTTCGGAGGGGGAAGATTATATATATA
AGTTAATAGAAAATAACAAAATAATTTATTCGATTAGTGGAAAAAAATTGACTTATAAAGG
AAAAAAATCTTTTTCAAAACATGCAATATTGAAACAGTTGAATGAAAAAGCAAACCAAGTT
AATTAAACAACCTATTTTATAGGATTTATAGGAAAGGAGAACAGCTGAATGAATATCCCTT
TTGTTGTAGAAACTGTGCTTCATGACGGCTTGTTAAAGTACAAATTTAAAAATAGTAAAAT
TCGCTCAATCACTACCAAGCCAGGTAAAAGCAAAGGGGCTATTTTTGCGTATCGCTCAAA
ATCAAGCATGATTGGCGGTCGTGGTGTTGTTCTGACTTCCGAGGAAGCGATTCAAGAAA
ATCAAGATACATTTACACATTGGACACCCAACGTTTATCGTTATGGAACGTATGCAGACG
AAAACCGTTCATACACGAAAGGACATTCTGAAAACAATTTAAGACAAATCAATACCTTCTT
TATTGATTTTGATATTCACACGGCAAAAGAAACTATTTCAGCAAGCGATATTTTAACAACC
GCTATTGATTTAGGTTTTATGCCTACTATGATTATCAAATCTGATAAAGGTTATCAAGCAT
ATTTTGTTTTAGAAACGCCAGTCTATGTGACTTCAAAATCAGAATTTAAATCTGTCAAAGC
AGCCAAAATAATTTCGCAAAATATCCGAGAATATTTTGGAAAGTCTTTGCCAGTTGATCTA
ACGTGTAATCATTTTGGTATTGCTCGCATACCAAGAACGGACAATGTAGAATTTTTTGATC
CTAATTACCGTTATTCTTTCAAAGAATGGCAAGATTGGTCTTTCAAACAAACAGATAATAA
GGGCTTTACTCGTTCAAGTCTAACGGTTTTAAGCGGTACAGAAGGCAAAAAACAAGTAG
ATGAACCCTGGTTTAATCTCTTATTGCACGAAACGAAATTTTCAGGAGAAAAGGGTTTAAT
AGGGCGTAATAACGTCATGTTTACCCTCTCTTTAGCCTACTTTAGTTCAGGCTATTCAATC
GAAACGTGCGAATATAATATGTTTGAGTTTAATAATCGATTAGATCAACCCTTAGAAGAAA
AAGAAGTAATCAAAATTGTTAGAAGTGCCTATTCAGAAAACTATCAAGGGGCTAATAGGG
AATACATTACCATTCTTTGCAAAGCTTGGGTATCAAGTGATTTAACCAGTAAAGATTTATT
TGTCCGTCAAGGGTGGTTTAAATTCAAGAAAAAAAGAAGCGAACGTCAACGTGTTCATTT
GTCAGAATGGAAAGAAGATTTAATGGCTTATATTAGCGAAAAAAGCGATGTATACAAGCC
TTATTTAGTGACGACCAAAAAAGAGATTAGAGAAGTGCTAGGCATTCCTGAACGGACATT
AGATAAATTGCTGAAGGTACTGAAGGCGAATCAGGAAATTTTCTTTAAGATTAAACCAGG
AAGAAATGGTGGCATTCAACTTGCTAGTGTTAAATCATTGTTGCTATCGATCATTAAAGTA
AAAAAAGAAGAAAAAGAAAGCTATATAAAGGCGCTGACAAATTCTTTTGACTTAGAGCAT
ACATTCATTCAAGAGACTTTAAACAAGCTAGCAGAACGCCCTAAAACGGACACACAACTC
GATTTGTTTAGCTATGATACAGGCTGAAAATAAAACCCGCACTATGCCATTACATTTATAT
CTATGATACGTGTTTGTTTTTTCTTTGCTGTTTAGCGAATGATTAGCAGAAATATACAGAG
TAAGATTTTAATTAATTATTAGGGGGAGAAGGAGAGAGTAGCCCGAAAACTTTTAGTTGG
CTTGGACTGAACGAAGTGAGGGAAAGGCTACTAAAACGTCGAGGGGCAGTGAGAGCGA
AGCGAACACTTGATTTTTTAATTTTCTATCTTTTATAGGTCATTAGAGTATACTTATTTGTC
CTATAAACTATTTAGCAGCATAATAGATTTATTGAATAGGTCATTTAAGTTGAGCATATTA
GAGGAGGAAAATCTTGGAGAAATATTTGAAGAACCCGATTACATGGATTGGATTAGTTCT
TGTGGTTACGTGGTTTTTAACTAAAAGTAGTGAATTTTTGATTTTTGGTGTGTGTGTCTTG
TTGTTAGTATTTGCTAGTCAAAGTGATTAAATA

pT1TR5AH Figure 1c (cont.)

GAATTCGATTAAGTCATCTTACCTCTTTTATTAGTTTTTTCTTATAATCTAATGATAACATTT
TTATAATTAATCTATAAACCATATCCCTCTTTGGAATCAAAATTTATTATCTACTCCTTTGTA
GATATGTTATAATACAAGTATCAGATCTGGGAGACCACAACGGTTTCCCACTAGAAATAA
TTTTGTTTAACTTTAGAAAGGAGATATACGCATGAAAAAAAAGATTATCTCAGCTATTTTAA
TGTCTACAGTCATACTTTCTGCTGCAGCCCCGTTGTCAGGTGTTTACGCCCTGGTCCCTT
CTCTTGGTGACCGGGAGAAGAGGGATAGCTTGTGTCCCCAAGGAAAGTATGTCCATTCT
AAGAACAATTCCATCTGCTGCACCAAGTGCCACAAAGGAACCTACTTGGTGAGTGACTG
TCCGAGCCCAGGGCGGGATACAGTCTGCAGGGAGTGTGAAAAGGGCACCTTTACGGCT
TCCCAGAATTACCTCAGGCAGTGTCTCAGTTGCAAGACATGTCGGAAAGAAATGTCCCA
GGTGGAGATCTCTCCTTGCCAAGCTGACAAGGACACGGTGTGTGGCTGTAAGGAGAAC
CAGTTCCAACGCTACCTGAGTGAGACACACTTCCAGTGCGTGGACTGCAGCCCCTGCTT
CAACGGCACCGTGACAATCCCCTGTAAGGAGACTCAGAACACCGTGTGTAACTGCCATG
CAGGGTTCTTTCTGAGAGAAAGTGAGTGCGTCCCTTGCAGCCACTGCAAGAAAAATGAG
GAGTGTATGAAGTTGTGCCTACCTCCTCCGCTTGCAAATGTCACAAACCCCCAGGACTC
AGGTACTGCGCATCATCATCATCATCATTAATAGACTAGTAGATCCGGCTGCTAACAAAG
CCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTT
GGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATG
ACCTGCAGGCAAGCTCTAGAATCGATACGATTTTGAAGTGGCAACAGATAAAAAAAAGCA
GTTTAAAATTGTTGCTGAACTTTTAAAACAAGCAAATACAATCATTGTCGCAACAGATAGC
GACAGAGAAGGCGAAAACATTGCCTGGTCGATCATTCATAAAGCAAATGCCTTTTCTAAA
GATAAAACGTATAAAAGACTATGGATCAATAGTTTAGAAAAAGATGTGATCCGTAGCGGT
TTTCAAAATTTGCAACCAGGAATGAATTACTATCCCTTTTATCAAGAAGCGCAAAAGAAAA
ACGAAATGATACACCAATCAGTGCAAAAAAAGATATAATGGGAGATAAGACGGTTCGTGT
TCGTGCTGACTTGCACCATATCATAAAAATCGAAACAGCAAAGAATGGCGGAAACGTAAA
AGAAGTTATGGAAATAAGACTTAGAAGCAAACTTAAGAGTGTGTTGATAGTGCAGTATCT
TAAAATTTTGTATAATAGGAATTGAAGTTAAATTAGATGCTAAAAATTTGTAATTAAGAAGG
AGTGATTACATGAACAAAAATATAAAATATTCTCAAAACTTTTTAACGAGTGAAAAAGTACT
CAACCAAATAATAAAACAATTGAATTTAAAAGAAACCGATACCGTTTACGAAATTGGAACA
GGTAAAGGGCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAA
TTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAATACTCGTGTCACTT
TAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGG
GAGTATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCATGCG
TCTGACATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTGGATATTCACCGA
ACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTGCTTAAGCTGCCAGC
GGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCATACC
ACAGATGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATC
GAGAATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCAATGAAACACGCCAAAG
TAAACAATTTAAGTACCGTTACTTATGAGCAAGTATTGTCTATTTTTAATAGTTATCTATTA
TTTAACGGGAGGAAATAATTCTATGAGTCGCTTTTGTAAATTTGGAAAGTTACACGTTACT
AAAGGGAATGTAGATAAATTATTAGGTATACTACTGACAGCTTCCAAGGAGCTAAAGAGG
TCCCTAGCGCTCTTATCATGGGGAAGCTCGGATCATATGCAAGACAAAATAAACTCGCAA
CAGCACTTGGAGAAATGGGACGAATCGAGAAAACCCTCTTTACGCTGGATTACATATCTA
ATAAAGCCGTAAGGAGACGGGTTCAAAAAGGTTTAAATAAAGGAGAAGCAATCAATGCA
TTAGCTAGAACTATATTTTTTGGACAACGTGGAGAATTTAGAGAACGTGCTCTCCAAGAC
CAGTTACAAAGAGCTAGTGCACTAAACATAATTATTAACGCTATAAGTGTGTGGAACACT
GTATATATGGAAAAAGCCGTAGAAGAATTAAAAGCAAGAGGAGAATTTAGAGAAGATTTA
ATGCCATATGCGTGGCCGTTAGGATGGGAACATATCAATTTTCTTGGAGAATACAAATTT
GAAGGATTACATGACACTGGGCAAATGAATTTACGTCCTTTACGTATAAAAGAGCCGTTT
TATTCTTAATATAACGGCTCTTTTTATAGAAAAAATCCTTAGCGTGGTTTTTTTCCGAAATG

pT1TR5AH (cont.) Figure 1c (cont.)

CTGGCGGTACCCCAAGAATTAGAAATGAGTAGATCAAATTATTCACGAATAGAATCAGGA
AAATCAGATCCAACCATAAAAACACTAGAACAAATTGCAAAGTTAACTAACTCAACGCTAG
TAGTGGATTTAATCCCAAATGAGCCAACAGAACCAGAGCCAGAAACAGAATCAGAACAA
GTAACATTGGATTTAGAAATGGAAGAAGAAAAAAGCAATGACTTCGTGTGAATAATGCAC
GAAATCGTTGCTTATTTTTTTTTAAAAGCGGTATACTAGATATAACGAAACAACGAACTGA
ATAGAAACGAAAAAAGAGCCATGACACATTTATAAAATGTTTGACGACATTTTATAAATGC
ATAGCCCGATAAGATTGCCAAACCAACGCTTATCAGTTAGTCAGATGAACTCTTCCCTCG
TAAGAAGTTATTTAATTAACTTTGTTTGAAGACGGTATATAACCGTACTATCATTATATAGG
GAAATCAGAGAGTTTTCAAGTATCTAAGCTACTGAATTTAAGAATTGTTAAGCAATCAATC
GGAAATCGTTTGATTGCTTTTTTTGTATTCATTTATAGAAGGTGGAGTTTGTATGAATCAT
GATGAATGTAAAACTTATATAAAAAATAGTTTATTGGAGATAAGAAAATTAGCAAATATCTA
TACACTAGAAACGTTTAAGAAAGAGTTAGAAAAGAGAAATATCTACTTAGAAACAAAATCA
GATAAGTATTTTTCTTCGGAGGGGGAAGATTATATATATAAGTTAATAGAAAATAACAAAA
TAATTTATTCGATTAGTGGAAAAAAATTGACTTATAAAGGAAAAAAATCTTTTTCAAAACAT
GCAATATTGAAACAGTTGAATGAAAAAGCAAACCAAGTTAATTAAACAACCTATTTTATAG
GATTTATAGGAAAGGAGAACAGCTGAATGAATATCCCTTTTGTTGTAGAAACTGTGCTTC
ATGACGGCTTGTTAAAGTACAAATTTAAAAATAGTAAAATTCGCTCAATCACTACCAAGCC
AGGTAAAAGCAAAGGGGCTATTTTTGCGTATCGCTCAAAATCAAGCATGATTGGCGGTC
GTGGTGTTGTTCTGACTTCCGAGGAAGCGATTCAAGAAAATCAAGATACATTTACACATT
GGACACCCAACGTTTATCGTTATGGAACGTATGCAGACGAAAACCGTTCATACACGAAA
GGACATTCTGAAAACAATTTAAGACAAATCAATACCTTCTTTATTGATTTTGATATTCACAC
GGCAAAAGAAACTATTTCAGCAAGCGATATTTTAACAACCGCTATTGATTTAGGTTTTATG
CCTACTATGATTATCAAATCTGATAAAGGTTATCAAGCATATTTTGTTTTAGAAACGCCAG
TCTATGTGACTTCAAAATCAGAATTTAAATCTGTCAAAGCAGCCAAAATAATTTCGCAAAA
TATCCGAGAATATTTTGGAAAGTCTTTGCCAGTTGATCTAACGTGTAATCATTTTGGTATT
GCTCGCATACCAAGAACGGACAATGTAGAATTTTTTGATCCTAATTACCGTTATTCTTTCA
AAGAATGGCAAGATTGGTCTTTCAAACAAACAGATAATAAGGGCTTTACTCGTTCAAGTC
TAACGGTTTTAAGCGGTACAGAAGGCAAAAAACAAGTAGATGAACCCTGGTTTAATCTCT
TATTGCACGAAACGAAATTTTCAGGAGAAAAGGGTTTAATAGGGCGTAATAACGTCATGT
TTACCCTCTCTTTAGCCTACTTTAGTTCAGGCTATTCAATCGAAACGTGCGAATATAATAT
GTTTGAGTTTAATAATCGATTAGATCAACCCTTAGAAGAAAAAGAAGTAATCAAAATTGTT
AGAAGTGCCTATTCAGAAAACTATCAAGGGGCTAATAGGGAATACATTACCATTCTTTGC
AAAGCTTGGGTATCAAGTGATTTAACCAGTAAAGATTTATTTGTCCGTCAAGGGTGGTTT
AAATTCAAGAAAAAAAGAAGCGAACGTCAACGTGTTCATTTGTCAGAATGGAAAGAAGAT
TTAATGGCTTATATTAGCGAAAAAAGCGATGTATACAAGCCTTATTTAGTGACGACCAAAA
AAGAGATTAGAGAAGTGCTAGGCATTCCTGAACGGACATTAGATAAATTGCTGAAGGTA
CTGAAGGCGAATCAGGAAATTTTCTTTAAGATTAAACCAGGAAGAAATGGTGGCATTCAA
CTTGCTAGTGTTAAATCATTGTTGCTATCGATCATTAAAGTAAAAAAAGAAGAAAAAGAAA
GCTATATAAAGGCGCTGACAAATTCTTTTGACTTAGAGCATACATTCATTCAAGAGACTTT
AAACAAGCTAGCAGAACGCCCTAAAACGGACACACAACTCGATTTGTTTAGCTATGATAC
AGGCTGAAAATAAAACCCGCACTATGCCATTACATTTATATCTATGATACGTGTTTGTTTT
TTCTTTGCTGTTTAGCGAATGATTAGCAGAAATATACAGAGTAAGATTTTAATTAATTATTA
GGGGGAGAAGGAGAGAGTAGCCCGAAAACTTTTAGTTGGCTTGGACTGAACGAAGTGA
GGGAAAGGCTACTAAAACGTCGAGGGGCAGTGAGAGCGAAGCGAACACTTGATTTTTTA
ATTTTCTATCTTTTATAGGTCATTAGAGTATACTTATTTGTCCTATAAACTATTTAGCAGCA
TAATAGATTTATTGAATAGGTCATTTAAGTTGAGCATATTAGAGGAGGAAAATCTTGGAGA
AATATTTGAAGAACCCGATTACATGGATTGGATTAGTTCTTGTGGTTACGTGGTTTTTAAC
TAAAAGTAGTGAATTTTTGATTTTTGGTGTGTGTGTCTTGTTGTTAGTATTTGCTAGTCAA
AGTGATTAAATA

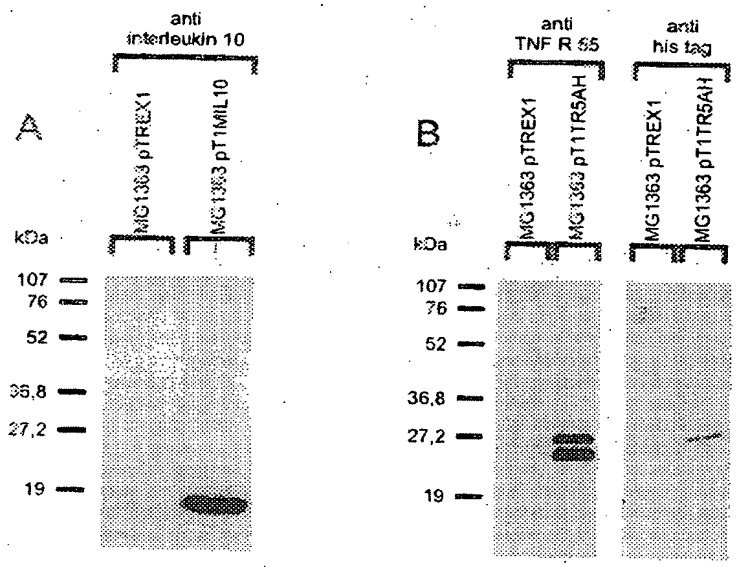
A
anti
interleukin 10
MG1363 pTREX1
MG1363 pT1ML10
kDa
107
76
52
36,8
27,2
19
B
anti
TNF R 55
anti
his tag
MG1363 pTREX1
MG1363 pT1TR5AH
MG1363 pTREX1
MG1363 pT1TR5AH
kDa
107
76
52
36,8
27,2
19

Figure 2

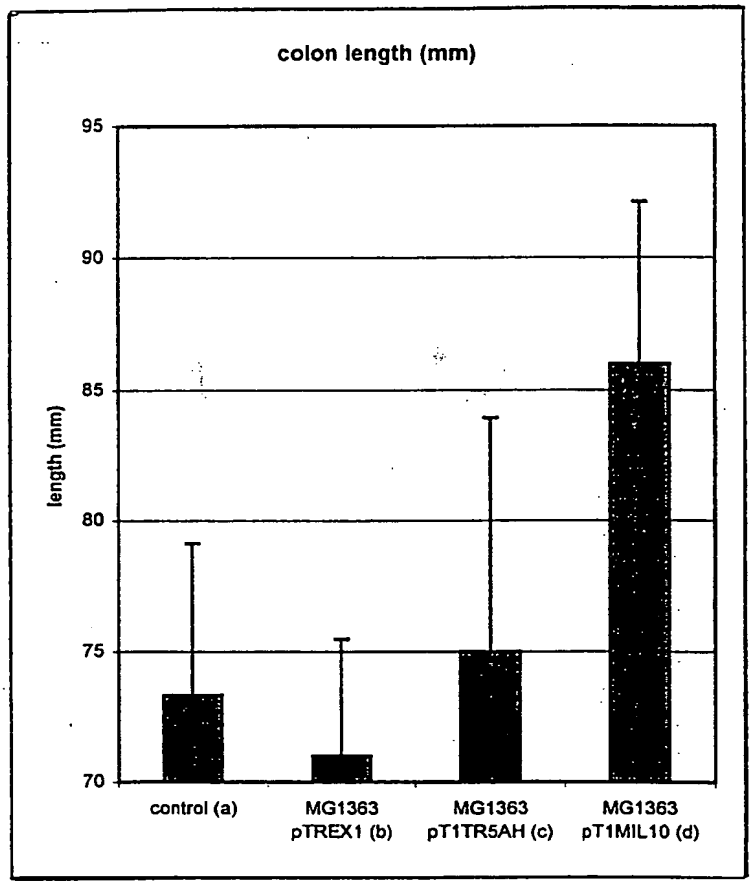
colon length (mm)
length (mm)
95
90
85
80
75
70
control (a)
MG1363 pTREX1 (b)
MG1363 pT1TR5AH (c)
MG1363 pT1MIL10 (d)

Figure 3

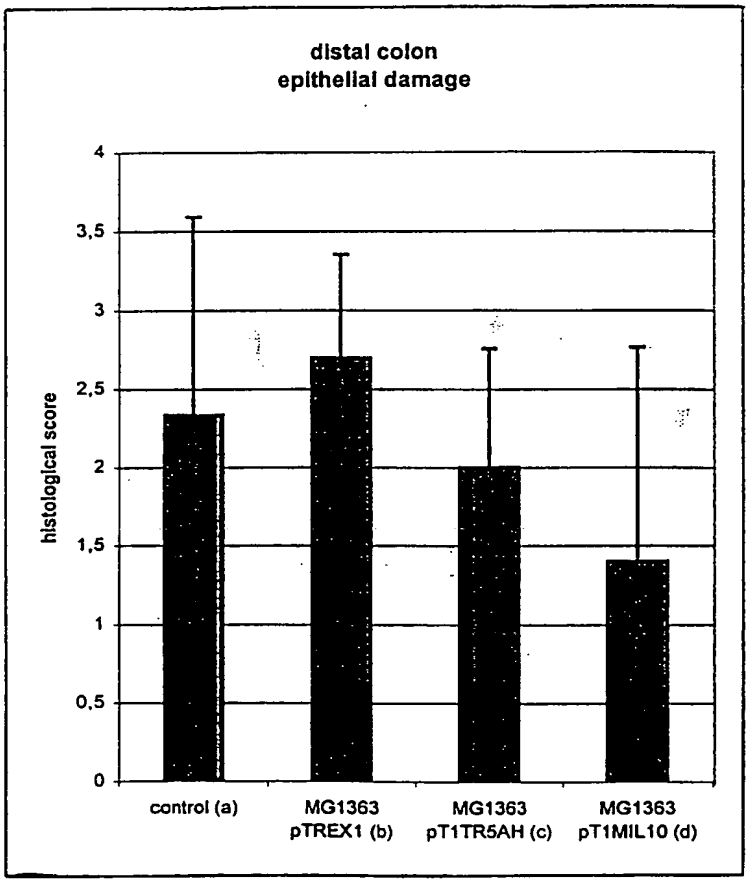
distal colon
epithelial damage
histological score
4
3,5
3
2,5
2
1,5
1
0,5
0
control (a)
MG1363 pTREX1 (b)
MG1363 pT1TR5AH (c)
MG1363 pT1MIL10 (d)

Figure 4

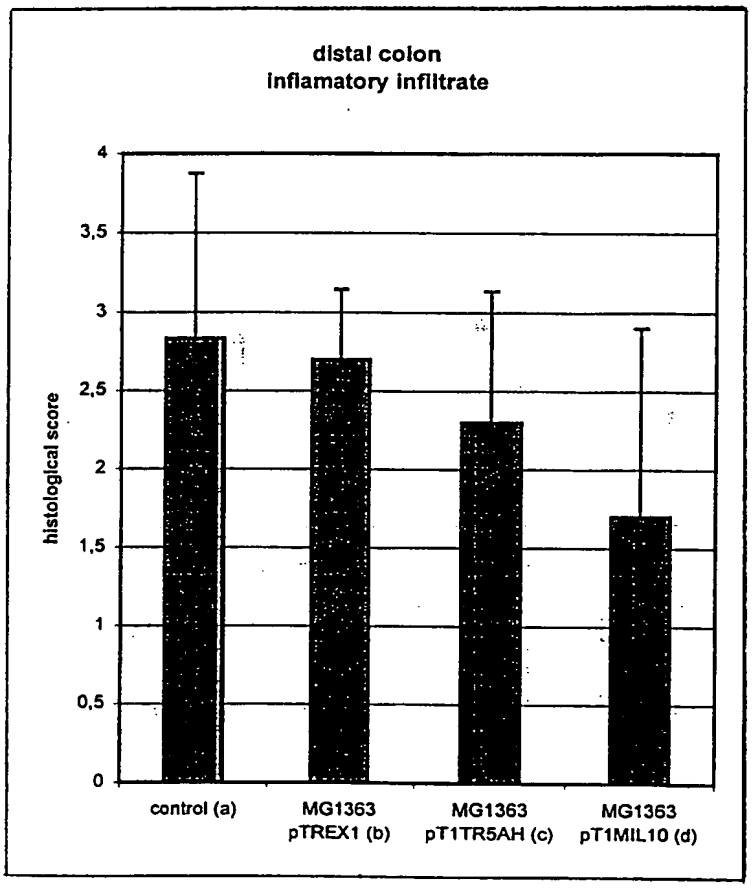
distal colon
inflamatory infiltrate
histological score
4
3,5
3
2,5
2
1,5
1
0,5
0
control (a)
MG1363 pTREX1 (b)
MG1363 pT1TR5AH (c)
MG1363 pT1MIL10 (d)

Figure 5

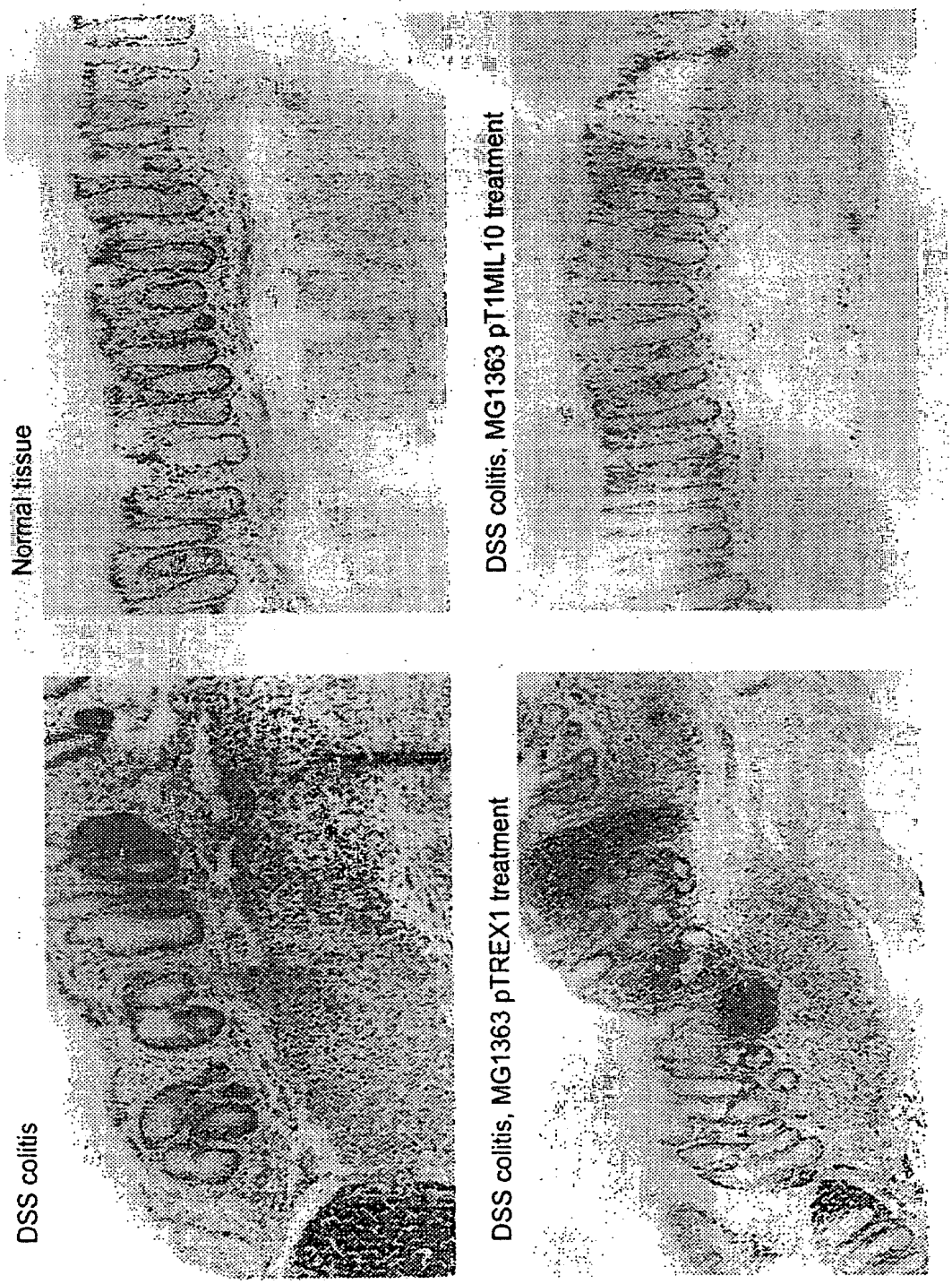
Normal tissue
DSS colitis, MG1363 pT1MIL10 treatment
DSS colitis
DSS colitis, MG1363 pTREX1 treatment

Figure 6

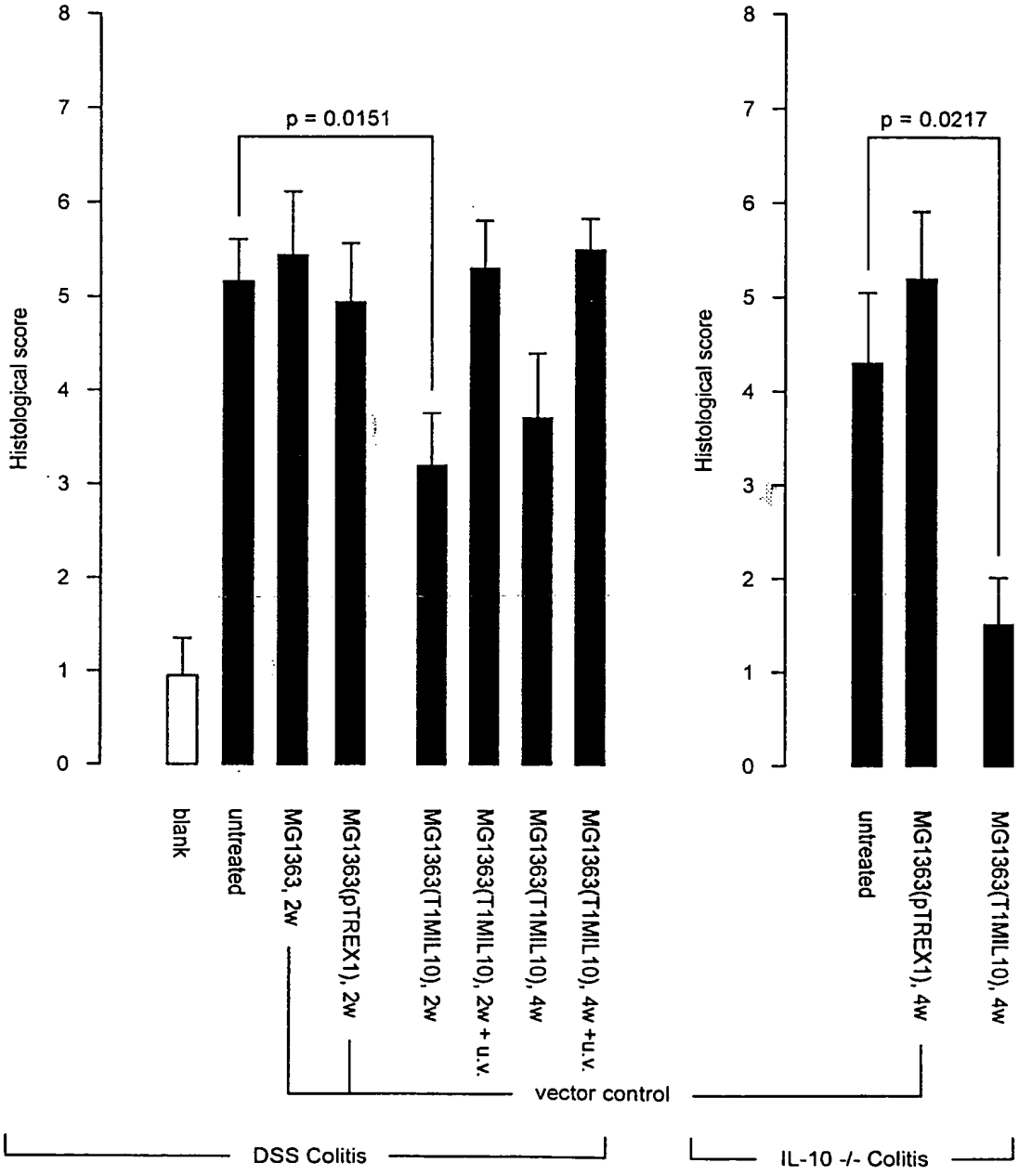
Histological score
8
7
6
5
4
3
2
1
0
p = 0.0151
p = 0.0217
blank
untreated
MG1363, 2w
MG1363(pTREX1), 2w
MG1363(T1MIL10), 2w
MG1363(T1MIL10), 2w + u.v.
MG1363(T1MIL10), 4w
MG1363(T1MIL10), 4w +u.v.
untreated
MG1363(pTREX1), 4w
MG1363(T1MIL10), 4w
vector control
DSS Colitis
IL-10 -/- Colitis

Figure 7

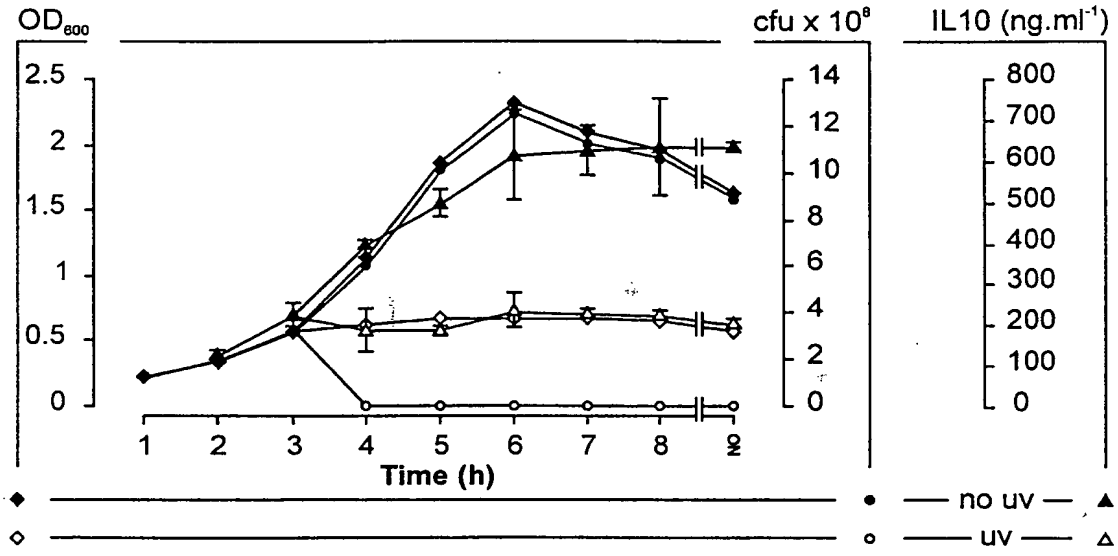
OD600
cfu x 10^8
IL10 (ng.ml^-1)
2.5
2
1.5
1
0.5
0
14
12
10
8
6
4
2
0
800
700
600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
Time (h)
no uv
uv

Figure 8

SEQUENCE LISTING

<110> VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOL

<120> USE OF CYTOKINE-PRODUCING LACTOCOCCUS STRAIN TO TREAT COLITIS

<130> V1/002-V023

<140>
<141>

<150> 98203529.7
<151> 1998-10-20

<160> 8

<170> PatentIn Ver. 2.1

<210> 1
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer used for obtaining the plasmid pT1MIL10

<400> 1
cagtacagcc gggaagacaa t 21

<210> 2
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer used for obtaining the plasmid pT1MIL10

<400> 2
gcactagtta gcttttcatt ttgat 25

<210> 3
<211> 21
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer used
for obtaining the plasmid pT1TR5AH

<400> 3
ctggtccctt ctcttggtga c 21

<210> 4
<211> 53
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer used
for obtaining the plasmid pT1TR5AH

<400> 4
ccactagtct attaatgatg atgatgatga tgcgcagtac ctgagtcctg ggg 53

<210> 5
<211> 5230
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid pTREX1

<400> 5
gaattcgatt aagtcatctt acctctttta ttagtttttt cttataatct aatgataaca 60
tttttataat taatctataa accatatccc tctttggaat caaaatttat tatctactcc 120
tttgtagata tgttataata caagtatcag atctgggaga ccacaacggt ttcccactag 180
aaataatttt gtttaacttt agaaaggaga tatacgcatg caggatatct ctagaatgga 240
tccggctgct aacaaagccc gaaaggaagc tgagttggct gctgccaccg ctgagcaata 300
actagcataa ccccttgggg cctctaaacg ggtcttgagg ggtttttttgc tgaaaggagg 360
aactatatcc ggatgacctg caggcaagct ctagaatcga tacgattttg aagtggcaac 420
agataaaaaa aagcagttta aaattgttgc tgaactttta aaacaagcaa atacaatcat 480
tgtcgcaaca gatagcgaca gagaaggcga aaacattgcc tggtcgatca ttcataaagc 540
aaatgccttt tctaaagata aaacgtataa aagactatgg atcaatagtt tagaaaaaga 600
tgtgatccgt agcggttttc aaaatttgca accaggaatg aattactatc ccttttatca 660
agaagcgcaa aagaaaaacg aaatgataca ccaatcagtg caaaaaaaga tataatggga 720
gataagacgg ttcgtgttcg tgctgacttg caccatatca taaaaatcga aacagcaaag 780
aatggcggaa acgtaaaaga agttatggaa ataagactta gaagcaaact taagagtgtg 840
ttgatagtgc agtatcttaa aattttgtat aataggaatt gaagttaaat tagatgctaa 900
aaatttgtaa ttaagaagga gtgattacat gaacaaaaat ataaaatatt ctcaaaactt 960

tttaacgagt gaaaaagtac tcaaccaaat aataaaacaa ttgaatttaa aagaaaccga 1020
taccgtttac gaaattggaa caggtaaagg gcatttaacg acgaaactgg ctaaaataag 1080
taaacaggta acgtctattg aattagacag tcatctattc aacttatcgt cagaaaaatt 1140
aaaactgaat actcgtgtca ctttaattca ccaagatatt ctacagtttc aattccctaa 1200
caaacagagg tataaaattg ttgggagtat tccttaccat ttaagcacac aaattattaa 1260
aaaagtggtt tttgaaagcc atgcgtctga catctatctg attgttgaag aaggattcta 1320
caagcgtacc ttggatattc accgaacact agggttgctc ttgcacactc aagtctcgat 1380
tcagcaattg cttaagctgc cagcggaatg ctttcatcct aaaccaaaag taaacagtgt 1440
cttaataaaa cttacccgcc ataccacaga tgttccagat aaatattgga agctatatac 1500
gtactttgtt tcaaaatggg tcaatcgaga atatcgtcaa ctgtttacta aaaatcagtt 1560
tcatcaagca atgaaacacg ccaaagtaaa caatttaagt accgttactt atgagcaagt 1620
attgtctatt tttaatagtt atctattatt taacgggagg aaataattct atgagtcgct 1680
tttgtaaatt tggaaagtta cacgttacta aagggaatgt agataaatta ttaggtatac 1740
tactgacagc ttccaaggag ctaaagaggt ccctagcgct cttatcatgg ggaagctcgg 1800
atcatatgca agacaaaata aactcgcaac agcacttgga gaaatgggac gaatcgagaa 1860
aaccctcttt acgctggatt acatatctaa taaagccgta aggagacggg ttcaaaaagg 1920
tttaaataaa ggagaagcaa tcaatgcatt agctagaact atatttttg gacaacgtgg 1980
agaatttaga gaacgtgctc tccaagacca gttacaaaga gctagtgcac taaacataat 2040
tattaacgct ataagtgtgt ggaacactgt atatatggaa aaagccgtag aagaattaaa 2100
agcaagagga gaatttagag aagatttaat gccatatgcg tggccgttag gatgggaaca 2160
tatcaatttt cttggagaat acaaatttga aggattacat gacactgggc aaatgaattt 2220
acgtccttta cgtataaaag agccgtttta ttcttaatat aacggctctt tttatagaaa 2280
aaatccttag cgtggttttt ttccgaaatg ctggcggtac cccaagaatt agaaatgagt 2340
agatcaaatt attcacgaat agaatcagga aaatcagatc caaccataaa aacactagaa 2400
caaattgcaa agttaactaa ctcaacgcta gtagtggatt taatcccaaa tgagccaaca 2460
gaaccagagc cagaaacaga atcagaacaa gtaacattgg atttagaaat ggaagaagaa 2520
aaaagcaatg acttcgtgtg aataatgcac gaaatcgttg cttatttttt tttaaaagcg 2580
gtatactaga tataacgaaa caacgaactg aatagaaacg aaaaaagagc catgacacat 2640
ttataaaatg tttgacgaca ttttataaat gcatagcccg ataagattgc caaaccaacg 2700
cttatcagtt agtcagatga actcttccct cgtaagaagt tatttaatta actttgtttg 2760
aagacggtat ataaccgtac tatcattata tagggaaatc agagagtttt caagtatcta 2820
agctactgaa tttaagaatt gttaagcaat caatcggaaa tcgtttgatt gctttttttg 2880
tattcattta tagaaggtgg agtttgtatg aatcatgatg aatgtaaaac ttatataaaa 2940
aatagtttat tggagataag aaaattagca aatatctata cactagaaac gtttaagaaa 3000
gagttagaaa agagaaatat ctacttagaa acaaaatcag ataagtattt ttcttcggag 3060
ggggaagatt atatatataa gttaatagaa aataacaaaa taatttattc gattagtgga 3120
aaaaaattga cttataaagg aaaaaaatct ttttcaaaac atgcaatatt gaaacagttg 3180
aatgaaaaag caaaccaagt taattaaaca acctatttta taggatttat aggaaaggag 3240
aacagctgaa tgaatatccc ttttgttgta gaaactgtgc ttcatgacgg cttgttaaag 3300
tacaaattta aaaatagtaa aattcgctca atcactacca agccaggtaa aagcaaaggg 3360
gctatttttg cgtatcgctc aaaatcaagc atgattggcg gtcgtggtgt tgttctgact 3420
tccgaggaag cgattcaaga aaatcaagat acatttacac attggacacc caacgtttat 3480
cgttatggaa cgtatgcaga cgaaaaccgt tcatacacga aaggacattc tgaaaacaat 3540
ttaagacaaa tcaatacctt ctttattgat tttgatattc acacggcaaa agaaactatt 3600
tcagcaagcg atattttaac aaccgctatt gatttaggtt ttatgcctac tatgattatc 3660
aaatctgata aaggttatca agcatatttt gttttagaaa cgccagtcta tgtgacttca 3720
aaatcagaat ttaaatctgt caaagcagcc aaaataattt cgcaaaatat ccgagaatat 3780
tttggaaagt ctttgccagt tgatctaacg tgtaatcatt ttggtattgc tcgcatacca 3840

agaacggaca atgtagaatt ttttgatcct aattaccgtt attctttcaa agaatggcaa 3900
gattggtctt tcaaacaaac agataataag ggctttactc gttcaagtct aacggtttta 3960
agcggtacag aaggcaaaaa acaagtagat gaaccctggt ttaatctctt attgcacgaa 4020
acgaaatttt caggagaaaa gggtttaata gggcgtaata acgtcatgtt taccctctct 4080
ttagcctact ttagttcagg ctattcaatc gaaacgtgcg aatataatat gtttgagttt 4140
aataatcgat tagatcaacc cttagaagaa aaagaagtaa tcaaaattgt tagaagtgcc 4200
tattcagaaa actatcaagg ggctaatagg gaatacatta ccattctttg caaagcttgg 4260
gtatcaagtg atttaaccag taaagattta tttgtccgtc aagggtggtt taaattcaag 4320
aaaaaaagaa gcgaacgtca acgtgttcat ttgtcagaat ggaaagaaga tttaatggct 4380
tatattagcg aaaaaagcga tgtatacaag ccttatttag tgacgaccaa aaaagagatt 4440
agagaagtgc taggcattcc tgaacggaca ttagataaat tgctgaaggt actgaaggcg 4500
aatcaggaaa ttttctttaa gattaaacca ggaagaaatg gtggcattca acttgctagt 4560
gttaaatcat tgttgctatc gatcattaaa gtaaaaaaag aagaaaaaga aagctatata 4620
aaggcgctga caaattcttt tgacttagag catacattca ttcaagagac tttaaacaag 4680
ctagcagaac gccctaaaac ggacacacaa ctcgatttgt ttagctatga tacaggctga 4740
aaataaaacc cgcactatgc cattacattt atatctatga tacgtgtttg ttttttcttt 4800
gctgtttagc gaatgattag cagaaatata cagagtaaga ttttaattaa ttattagggg 4860
gagaaggaga gagtagcccg aaaactttta gttggcttgg actgaacgaa gtgagggaaa 4920
ggctactaaa acgtcgaggg gcagtgagag cgaagcgaac acttgatttt ttaatttttct 4980
atcttttata ggtcattaga gtatacttat ttgtcctata aactatttag cagcataata 5040
gatttattga ataggtcatt taagttgagc atattagagg aggaaaatct tggagaaata 5100
tttgaagaac ccgattacat ggattggatt agttcttgtg gttacgtggt ttttaactaa 5160
aagtagtgaa tttttgattt ttggtgtgtg tgtcttgttg ttagtatttg ctagtcaaag 5220
tgattaaata 5230

<210> 6
<211> 5906
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plamsid pT1NX

<400> 6
gaattcgatt aagtcatctt acctctttta ttagtttttt cttataatct aatgataaca 60
tttttataat taatctataa accatatccc tctttggaat caaaatttat tatctactcc 120
tttgtagata tgttataata caagtatcag atctgggaga ccacaacggt ttcccactag 180
aaataatttt gtttaacttt agaaaggaga tatacgcatg aaaaaaaaga ttatctcagc 240
tattttaatg tctacagtca tactttctgc tgcagccccg ttgtcaggtg tttacgccgg 300
cgacggatcc aaaagaggaa gacaataaca agcctggcaa agaagacaat aacaagcctg 360
gcaaagaaga caataacaag cctggcaaag aagacaacaa caagcctggc aaagaagaca 420
acaacaagcc tggtaaagaa gacaacaaca agcctggcaa agaagacggc aacaagcctg 480
gtaaagaaga caacaaaaaa cctggtaaag aagatggcaa caagcctggt aaagaagaca 540
acaaaaaacc tggtaaagaa gacggcaaca agcctggcaa agaagatggc aacaaacctg 600
gtaaagaaga tggtaacgga gtacatgtcg ttaaacctgg tgatacagta aatgacattg 660
caaaagcaaa cggcactact gctgacaaaa ttgctgcaga taacaaatta gctgataaaa 720
acatgatcaa acctggtcaa gaacttgttg ttgataagaa gcaaccagca aaccatgcag 780

atgctaacaa agctcaagca ttaccagaaa ctggcgaaga aaatccattc atcggtacaa 840
ctgtatttgg tggattatca ttagccttag gtgcagcgtt attagctgga cgtcgtcgcg 900
aactataact agtagatccg gctgctaaca aagcccgaaa ggaagctgag ttggctgctg 960
ccaccgctga gcaataacta gcataacccc ttggggcctc taaacgggtc ttgaggggtt 1020
ttttgctgaa aggaggaact atatccggat gacctgcagg caagctctag aatcgatacg 1080
attttgaagt ggcaacagat aaaaaaaagc agtttaaaat tgttgctgaa cttttaaaac 1140
aagcaaatac aatcattgtc gcaacagata gcgacagaga aggcgaaaac attgcctggt 1200
cgatcattca taaagcaaat gccttttcta aagataaaac gtataaaaga ctatggatca 1260
atagtttaga aaaagatgtg atccgtagcg gttttcaaaa tttgcaacca ggaatgaatt 1320
actatccctt ttatcaagaa gcgcaaaaga aaaacgaaat gatacaccaa tcagtgcaaa 1380
aaaagatata atgggagata agacggttcg tgttcgtgct gacttgcacc atatcataaa 1440
aatcgaaaca gcaaagaatg gcggaaacgt aaaagaagtt atggaaataa gacttagaag 1500
caaacttaag agtgtgttga tagtgcagta tcttaaaatt ttgtataata ggaattgaag 1560
ttaaattaga tgctaaaaat ttgtaattaa gaaggagtga ttacatgaac aaaaatataa 1620
aatattctca aaacttttta acgagtgaaa aagtactcaa ccaaataata aaacaattga 1680
atttaaaaga aaccgatacc gtttacgaaa ttggaacagg taaagggcat ttaacgacga 1740
aactggctaa aataagtaaa caggtaacgt ctattgaatt agacagtcat ctattcaact 1800
tatcgtcaga aaaattaaaa ctgaatactc gtgtcacttt aattcaccaa gatattctac 1860
agtttcaatt ccctaacaaa cagaggtata aaattgttgg gagtattcct taccatttaa 1920
gcacacaaat tattaaaaaa gtggtttttg aaagccatgc gtctgacatc tatctgattg 1980
ttgaagaagg attctacaag cgtaccttgg atattcaccg aacactaggg ttgctcttgc 2040
acactcaagt ctcgattcag caattgctta agctgccagc ggaatgcttt catcctaaac 2100
caaaagtaaa cagtgtctta ataaaactta cccgccatac cacagatgtt ccagataaat 2160
attggaagct atatacgtac tttgtttcaa aatgggtcaa tcgagaatat cgtcaactgt 2220
ttactaaaaa tcagtttcat caagcaatga aacacgccaa agtaaacaat ttaagtaccg 2280
ttacttatga gcaagtattg tctattttta atagttatct attatttaac gggaggaaat 2340
aattctatga gtcgcttttg taaatttgga aagttacacg ttactaaagg gaatgtagat 2400
aaattattag gtatactact gacagcttcc aaggagctaa agaggtccct agcgctctta 2460
tcatggggaa gctcggatca tatgcaagac aaaataaact cgcaacagca cttggagaaa 2520
tgggacgaat cgagaaaacc ctctttacgc tggattacat atctaataaa gccgtaagga 2580
gacgggttca aaaaggttta aataaaggag aagcaatcaa tgcattagct agaactatat 2640
tttttggaca acgtggagaa tttagagaac gtgctctcca agaccagtta caaagagcta 2700
gtgcactaaa cataattatt aacgctataa gtgtgtggaa cactgtatat atggaaaaag 2760
ccgtagaaga attaaaagca agaggagaat ttagagaaga tttaatgcca tatgcgtggc 2820
cgttaggatg ggaacatatc aatttttcttg gagaatacaa atttgaagga ttacatgaca 2880
ctgggcaaat gaatttacgt cctttacgta taaaagagcc gttttattct taatataacg 2940
gctcttttta tagaaaaaat ccttagcgtg gtttttttcc gaaatgctgg cggtacccca 3000
agaattagaa atgagtagat caaattattc acgaatagaa tcaggaaaat cagatccaac 3060
cataaaaaca ctagaacaaa ttgcaaagtt aactaactca acgctagtag tggatttaat 3120
cccaaatgag ccaacagaac cagagccaga aacagaatca gaacaagtaa cattggattt 3180
agaaatggaa gaagaaaaaa gcaatgactt cgtgtgaata atgcacgaaa tcgttgctta 3240
tttttttttа aaagcggtat actagatata acgaaacaac gaactgaata gaaacgaaaa 3300
aagagccatg acacatttat aaaatgtttg acgacatttt ataaatgcat agcccgataa 3360
gattgccaaa ccaacgctta tcagttagtc agatgaactc ttccctcgta agaagttatt 3420
taattaactt tgtttgaaga cggtatataa ccgtactatc attatatagg gaaatcagag 3480
agttttcaag tatctaagct actgaattta agaattgtta agcaatcaat cggaaatcgt 3540
ttgattgctt tttttgtatt catttataga aggtggagtt tgtatgaatc atgatgaatg 3600
taaaacttat ataaaaaata gtttattgga gataagaaaa ttagcaaata tctatacact 3660

agaaacgttt aagaaagagt tagaaaagag aaatatctac ttagaaacaa aatcagataa 3720
gtatttttct tcggaggggg aagattatat atataagtta atagaaaata acaaaataat 3780
ttattcgatt agtggaaaaa aattgactta taaaggaaaa aaatcttttt caaaacatgc 3840
aatattgaaa cagttgaatg aaaaagcaaa ccaagttaat taaacaacct attttatagg 3900
atttatagga aaggagaaca gctgaatgaa tatccctttt gttgtagaaa ctgtgcttca 3960
tgacggcttg ttaaagtaca aatttaaaaa tagtaaaatt cgctcaatca ctaccaagcc 4020
aggtaaaagc aaaggggcta tttttgcgta tcgctcaaaa tcaagcatga ttggcggtcg 4080
tggtgttgtt ctgacttccg aggaagcgat tcaagaaaat caagatacat ttacacattg 4140
gacacccaac gtttatcgtt atggaacgta tgcagacgaa aaccgttcat acacgaaagg 4200
acattctgaa aacaatttaa gacaaatcaa taccttcttt attgattttg atattcacac 4260
ggcaaaagaa actatttcag caagcgatat tttaacaacc gctattgatt taggttttat 4320
gcctactatg attatcaaat ctgataaagg ttatcaagca tattttgttt tagaaacgcc 4380
agtctatgtg acttcaaaat cagaatttaa atctgtcaaa gcagccaaaa taatttcgca 4440
aaatatccga gaatattttg gaaagtcttt gccagttgat ctaacgtgta atcattttgg 4500
tattgctcgc ataccaagaa cggacaatgt agaatttttt gatcctaatt accgttattc 4560
tttcaaagaa tggcaagatt ggtctttcaa acaaacagat aataagggct ttactcgttc 4620
aagtctaacg gttttaagcg gtacagaagg caaaaaacaa gtagatgaac cctggtttaa 4680
tctcttattg cacgaaacga aattttcagg agaaaagggt ttaatagggc gtaataacgt 4740
catgtttacc ctctctttag cctactttag ttcaggctat tcaatcgaaa cgtgcgaata 4800
taatatgttt gagtttaata atcgattaga tcaaccctta gaagaaaaag aagtaatcaa 4860
aattgttaga agtgcctatt cagaaaacta tcaaggggct aatagggaat acattaccat 4920
tctttgcaaa gcttgggtat caagtgattt aaccagtaaa gatttatttg tccgtcaagg 4980
gtggtttaaa ttcaagaaaa aaagaagcga acgtcaacgt gttcatttgt cagaatggaa 5040
agaagattta atggcttata ttagcgaaaa aagcgatgta tacaagcctt atttagtgac 5100
gaccaaaaaa gagattagag aagtgctagg cattcctgaa cggacattag ataaattgct 5160
gaaggtactg aaggcgaatc aggaaatttt ctttaagatt aaaccaggaa gaaatggtgg 5220
cattcaactt gctagtgtta aatcattgtt gctatcgatc attaaagtaa aaaaagaaga 5280
aaaagaaagc tatataaagg cgctgacaaa ttcttttgac ttagagcata cattcattca 5340
agagactttá aacaagctag cagaacgccc taaaacggac acacaactcg atttgtttag 5400
ctatgataca ggctgaaaat aaaaccccgca ctatgccatt acatttatat ctatgatacg 5460
tgtttgtttt ttctttgctg tttagcgaat gattagcaga aatatacaga gtaagatttt 5520
aattaattat tagggggaga aggagagagt agcccgaaaa cttttagttg gcttggactg 5580
aacgaagtga gggaaaggct actaaaacgt cgaggggcag tgagagcgaa gcgaacactt 5640
gatttttaa ttttctatct tttataggtc attagagtat acttatttgt cctataaact 5700
atttagcagc ataatagatt tattgaatag gtcatttaag ttgagcatat tagaggagga 5760
aaatcttgga gaaatatttg aagaacccga ttacatggat tggattagtt cttgtggtta 5820
cgtggttttt aactaaaagt agtgaatttt tgatttttgg tgtgtgtgtc ttgttgttag 5880
tatttgctag tcaaagtgat taaata 5906

<210> 7
<211> 5770
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid
pT1MIL10

<400> 7

gaattcgatt aagtcatctt acctctttta ttagtttttt cttataatct aatgataaca 60
tttttataat taatctataa accatatccc tctttggaat caaaatttat tatctactcc 120
tttgtagata tgttataata caagtatcag atctgggaga ccacaacggt ttcccactag 180
aaataatttt gtttaacttt agaaaggaga tatacgcatg aaaaaaaaga ttatctcagc 240
tattttaatg tctacagtca tactttctgc tgcagccccg ttgtcaggtg tttacgccca 300
gtacagccgg gaagacaata actgcaccca cttcccagtc ggccagagcc acatgctcct 360
agagctgcgg actgccttca gccaggtgaa gactttcttt caaacaaagg accagctgga 420
caacatactg ctaaccgact ccttaatgca ggactttaag ggttacttgg gttgccaagc 480
cttatcggaa atgatccagt tttacctggt agaagtgatg ccccaggcag agaagcatgg 540
cccagaaatc aaggagcatt tgaattccct gggtgagaag ctgaagaccc tcaggatgcg 600
gctgaggcgc tgtcatcgat ttctcccctg tgaaaataag agcaaggcag tggagcaggt 660
gaagagtgat tttaataagc tccaagacca aggtgtctac aaggccatga atgaatttga 720
catcttcatc aactgcatag aagcatacat gatgatcaaa atgaaaagct aactagtaga 780
tccggctgct aacaaagccc gaaaggaagc tgagttggct gctgccaccg ctgagcaata 840
actagcataa ccccttgggg cctctaaacg ggtcttgagg ggtttttgc tgaaaggagg 900
aactatatcc ggatgacctg caggcaagct ctagaatcga tacgattttg aagtggcaac 960
agataaaaaa aagcagttta aaattgttgc tgaactttta aaacaagcaa atacaatcat 1020
tgtcgcaaca gatagcgaca gagaaggcga aaacattgcc tggtcgatca ttcataaagc 1080
aaatgccttt tctaaagata aaacgtataa aagactatgg atcaatagtt tagaaaaaga 1140
tgtgatccgt agcggttttc aaaatttgca accaggaatg aattactatc ccttttatca 1200
agaagcgcaa aagaaaaacg aaatgataca ccaatcagtg caaaaaaaga tataatggga 1260
gataagacgg ttcgtgttcg tgctgacttg caccatatca taaaaatcga aacagcaaag 1320
aatggcggaa acgtaaaaga agttatggaa ataagactta gaagcaaact taagagtgtg 1380
ttgatagtgc agtatcttaa aattttgtat aataggaatt gaagttaaat tagatgctaa 1440
aaatttgtaa ttaagaagga gtgattacat gaacaaaaat ataaaatatt ctcaaaactt 1500
tttaacgagt gaaaaagtac tcaaccaaat aataaaacaa ttgaatttaa aagaaaccga 1560
taccgtttac gaaattggaa caggtaaagg gcatttaacg acgaaactgg ctaaaataag 1620
taaacaggta acgtctattg aattagacag tcatctattc aacttatcgt cagaaaaatt 1680
aaaactgaat actcgtgtca ctttaattca ccaagatatt ctacagtttc aattccctaa 1740
caaacagagg tataaaattg ttgggagtat tccttaccat ttaagcacac aaattattaa 1800
aaaagtggtt tttgaaagcc atgcgtctga catctatctg attgttgaag aaggattcta 1860
caagcgtacc ttggatattc accgaacact agggttgctc ttgcacactc aagtctcgat 1920
tcagcaattg cttaagctgc cagcggaatg ctttcatcct aaaccaaaag taaacagtgt 1980
cttaataaaa cttacccgcc ataccacaga tgttccagat aaatattgga agctatatac 2040
gtactttgtt tcaaaatggg tcaatcgaga atatcgtcaa ctgtttacta aaaatcagtt 2100
tcatcaagca atgaaacacg ccaaagtaaa caatttaagt accgttactt atgagcaagt 2160
attgtctatt tttaatagtt atctattatt taacgggagg aaataattct atgagtcgct 2220
tttgtaaatt tggaaagtta cacgttacta aagggaatgt agataaatta ttaggtatac 2280
tactgacagc ttccaaggag ctaaagaggt ccctagcgct cttatcatgg ggaagctcgg 2340
atcatatgca agacaaaata aactcgcaac agcacttgga gaaatgggac gaatcgagaa 2400
aaccctcttt acgctggatt acatatctaa taaagccgta aggagacggg ttcaaaaagg 2460
tttaaataaa ggagaagcaa tcaatgcatt agctagaact atatttttg gacaacgtgg 2520
agaatttaga gaacgtgctc tccaagacca gttacaaaga gctagtgcac taaacataat 2580
tattaacgct ataagtgtgt ggaacactgt atatatggaa aaagccgtag aagaattaaa 2640
agcaagagga gaatttagag aagatttaat gccatatgcg tggccgttag gatgggaaca 2700
tatcaatttt cttggagaat acaaatttga aggattacat gacactgggc aaatgaattt 2760

acgtccttta cgtataaaag agccgtttta ttcttaatat aacggctctt tttatagaaa 2820
aaatccttag cgtggttttt ttccgaaatg ctggcggtac cccaagaatt agaaatgagt 2880
agatcaaatt attcacgaat agaatcagga aaatcagatc caaccataaa aacactagaa 2940
caaattgcaa agttaactaa ctcaacgcta gtagtggatt taatcccaaa tgagccaaca 3000
gaaccagagc cagaaacaga atcagaacaa gtaacattgg atttagaaat ggaagaagaa 3060
aaaagcaatg acttcgtgtg aataatgcac gaaatcgttg cttatttttt tttaaaagcg 3120
gtatactaga tataacgaaa caacgaactg aatagaaacg aaaaaagagc catgacacat 3180
ttataaaatg tttgacgaca ttttataaat gcatagcccg ataagattgc caaaccaacg 3240
cttatcagtt agtcagatga actcttccct cgtaagaagt tatttaatta actttgtttg 3300
aagacggtat ataaccgtac tatcattata tagggaaatc agagagtttt caagtatcta 3360
agctactgaa tttaagaatt gttaagcaat caatcggaaa tcgtttgatt gctttttttg 3420
tattcattta tagaaggtgg agtttgtatg aatcatgatg aatgtaaaac ttatataaaa 3480
aatagtttat tggagataag aaaattagca aatatctata cactagaaac gtttaagaaa 3540
gagttagaaa agagaaatat ctacttagaa acaaaatcag ataagtattt ttcttcggag 3600
ggggaagatt atatatataa gttaatagaa aataacaaaa taatttattc gattagtgga 3660
aaaaaattga cttataaagg aaaaaaatct ttttcaaaac atgcaatatt gaaacagttg 3720
aatgaaaaag caaaccaagt taattaaaca acctatttta taggatttat aggaaaggag 3780
aacagctgaa tgaatatccc ttttgttgta gaaactgtgc ttcatgacgg cttgttaaag 3840
tacaaattta aaaatagtaa aattcgctca atcactacca agccaggtaa aagcaaaggg 3900
gctatttttg cgtatcgctc aaaatcaagc atgattggcg gtcgtggtgt tgttctgact 3960
tccgaggaag cgattcaaga aaatcaagat acatttacac attggacacc caacgtttat 4020
cgttatggaa cgtatgcaga cgaaaaccgt tcatacacga aaggacattc tgaaaacaat 4080
ttaagacaaa tcaatacctt ctttattgat tttgatattc acacggcaaa agaaactatt 4140
tcagcaagcg atattttaac aaccgctatt gatttaggtt ttatgcctac tatgattatc 4200
aaatctgata aaggttatca agcatatttt gttttagaaa cgccagtcta tgtgacttca 4260
aaatcagaat ttaaatctgt caaagcagcc aaaataattt cgcaaaatat ccgagaatat 4320
tttggaaagt ctttgccagt tgatctaacg tgtaatcatt ttggtattgc tcgcatacca 4380
agaacggaca atgtagaatt ttttgatcct aattaccgtt attctttcaa agaatggcaa 4440
gattggtctt tcaaacaaac agataataag ggctttactc gttcaagtct aacggtttta 4500
agcggtacag aaggcaaaaa acaagtagat gaaccctggt ttaatctctt attgcacgaa 4560
acgaaatttt caggagaaaa gggtttaata gggcgtaata acgtcatgtt taccctctct 4620
ttagcctact ttagttcagg ctattcaatc gaaacgtgcg aatataatat gtttgagttt 4680
aataatcgat tagatcaacc cttagaagaa aaagaagtaa tcaaaattgt tagaagtgcc 4740
tattcagaaa actatcaagg ggctaatagg gaatacatta ccattctttg caaagcttgg 4800
gtatcaagtg atttaaccag taaagattta tttgtccgtc aagggtggtt taaattcaag 4860
aaaaaaagaa gcgaacgtca acgtgttcat ttgtcagaat ggaaagaaga tttaatggct 4920
tatattagcg aaaaaagcga tgtatacaag ccttatttag tgacgaccaa aaaagagatt 4980
agagaagtgc taggcattcc tgaacggaca ttagataaat tgctgaaggt actgaaggcg 5040
aatcaggaaa ttttctttaa gattaaacca ggaagaaatg gtggcattca acttgctagt 5100
gttaaatcat tgttgctatc gatcattaaa gtaaaaaaag aagaaaaaga aagctatata 5160
aaggcgctga caaattcttt tgacttagag catacattca ttcaagagac tttaaacaag 5220
ctagcagaac gccctaaaac ggacacacaa ctcgatttgt ttagctatga tacaggctga 5280
aaataaaacc cgcactatgc cattacattt atatctatga tacgtgtttg ttttttcttt 5340
gctgtttagc gaatgattag cagaaatata cagagtaaga ttttaattaa ttattagggg 5400
gagaaggaga gagtagcccg aaaactttta gttggcttgg actgaacgaa gtgagggaaa 5460
ggctactaaa acgtcgaggg gcagtgagag cgaagcgaac acttgatttt ttaatttttct 5520
atcttttata ggtcattaga gtatacttat ttgtcctata aactatttag cagcataata 5580
gatttattga ataggtcatt taagttgagc atattagagg aggaaaatct tggagaaata 5640

tttgaagaac ccgattacat ggattggatt agttcttgtg gttacgtggt ttttaactaa 5700
aagtagtgaa tttttgattt ttggtgtgtg tgtcttgttg ttagtatttg ctagtcaaag 5760
tgattaaata 5770

<210> 8
<211> 5870
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid
pT1TR5AH

<400> 8
gaattcgatt aagtcatctt acctctttta ttagtttttt cttataatct aatgataaca 60
tttttataat taatctataa accatatccc tctttggaat caaaatttat tatctactcc 120
tttgtagata tgttataata caagtatcag atctgggaga ccacaacggt ttcccactag 180
aaataatttt gtttaacttt agaaaggaga tatacgcatg aaaaaaaaga ttatctcagc 240
tattttaatg tctacagtca tactttctgc tgcagccccg ttgtcaggtg tttacgccct 300
ggtcccttct cttggtgacc gggagaagag ggatagcttg tgtccccaag gaaagtatgt 360
ccattctaag aacaattcca tctgctgcac caagtgccac aaaggaacct acttggtgag 420
tgactgtccg agcccagggc gggatacagt ctgcagggag tgtgaaaagg gcacctttac 480
ggcttcccag aattacctca ggcagtgtct cagttgcaag acatgtcgga aagaaatgtc 540
ccaggtggag atctctcctt gccaagctga caaggacacg gtgtgtggct gtaaggagaa 600
ccagttccaa cgctacctga gtgagacaca cttccagtgc gtggactgca gcccctgctt 660
caacggcacc gtgacaatcc cctgtaagga gactcagaac accgtgtgta actgccatgc 720
agggttcttt ctgagagaaa gtgagtgcgt cccttgcagc cactgcaaga aaaatgagga 780
gtgtatgaag ttgtgcctac ctcctccgct tgcaaatgtc acaaaccccc aggactcagg 840
tactgcgcat catcatcatc atcattaata gactagtaga tccggctgct aacaaagccc 900
gaaaggaagc tgagttggct gctgccaccg ctgagcaata actagcataa ccccttgggg 960
cctctaaacg ggtcttgagg ggtttttgc tgaaaggagg aactatatcc ggatgacctg 1020
caggcaagct ctagaatcga tacgattttg aagtggcaac agataaaaaa aagcagttta 1080
aaattgttgc tgaactttta aaacaagcaa atacaatcat tgtcgcaaca gatagcgaca 1140
gagaaggcga aaacattgcc tggtcgatca ttcataaagc aaatgccttt tctaaagata 1200
aaacgtataa aagactatgg atcaatagtt tagaaaaaga tgtgatccgt agcggttttc 1260
aaaatttgca accaggaatg aattactatc ccttttatca agaagcgcaa aagaaaaacg 1320
aaatgataca ccaatcagtg caaaaaaaga tataatggga gataagacgg ttcgtgttcg 1380
tgctgacttg caccatatca taaaaatcga aacagcaaag aatggcggaa acgtaaaaga 1440
agttatggaa ataagactta gaagcaaact taagagtgtg ttgatagtgc agtatcttaa 1500
aattttgtat aataggaatt gaagttaaat tagatgctaa aaatttgtaa ttaagaagga 1560
gtgattacat gaacaaaaat ataaaatatt ctcaaaactt tttaacgagt gaaaaagtac 1620
tcaaccaaat aataaaacaa ttgaatttaa aagaaaccga taccgtttac gaaattggaa 1680
caggtaaagg gcatttaacg acgaaactgg ctaaaataag taaacaggta acgtctattg 1740
aattagacag tcatctattc aacttatcgt cagaaaaatt aaaactgaat actcgtgtca 1800
ctttaattca ccaagatatt ctacagtttc aattccctaa caaacagagg tataaaattg 1860
ttgggagtat tccttaccat ttaagcacac aaattattaa aaaagtggtt tttgaaagcc 1920
atgcgtctga catctatctg attgttgaag aaggattcta caagcgtacc ttggatattc 1980

accgaacact agggttgctc ttgcacactc aagtctcgat tcagcaattg cttaagctgc 2040
cagcggaatg ctttcatcct aaaccaaaag taaacagtgt cttaataaaa cttacccgcc 2100
ataccacaga tgttccagat aaatattgga agctatatac gtactttgtt tcaaaatggg 2160
tcaatcgaga atatcgtcaa ctgtttacta aaaatcagtt tcatcaagca atgaaacacg 2220
ccaaagtaaa caatttaagt accgttactt atgagcaagt attgtctatt tttaatagtt 2280
atctattatt taacgggagg aaataattct atgagtcgct tttgtaaatt tggaaagtta 2340
cacgttacta aagggaatgt agataaatta ttaggtatac tactgacagc ttccaaggag 2400
ctaaagaggt ccctagcgct cttatcatgg ggaagctcgg atcatatgca agacaaaata 2460
aactcgcaac agcacttgga gaaatgggac gaatcgagaa aaccctcttt acgctggatt 2520
acatatctaa taaagccgta aggagacggg ttcaaaaagg tttaaataaa ggagaagcaa 2580
tcaatgcatt agctagaact atatttttg gacaacgtgg agaatttaga gaacgtgctc 2640
tccaagacca gttacaaaga gctagtgcac taaacataat tattaacgct ataagtgtgt 2700
ggaacactgt atatatggaa aaagccgtag aagaattaaa agcaagagga gaatttagag 2760
aagatttaat gccatatgcg tggccgttag gatgggaaca tatcaatttt cttggagaat 2820
acaaatttga aggattacat gacactgggc aaatgaattt acgtccttta cgtataaaag 2880
agccgtttta ttcttaatat aacggctctt tttatagaaa aaatccttag cgtggttttt 2940
ttccgaaatg ctggcggtac cccaagaatt agaaatgagt agatcaaatt attcacgaat 3000
agaatcagga aaatcagatc caaccataaa aacactagaa caaattgcaa agttaactaa 3060
ctcaacgcta gtagtggatt taatcccaaa tgagccaaca gaaccagagc cagaaacaga 3120
atcagaacaa gtaacattgg atttagaaat ggaagaagaa aaaagcaatg acttcgtgtg 3180
aataatgcac gaaatcgttg cttatttttt tttaaaagcg gtatactaga tataacgaaa 3240
caacgaactg aatagaaacg aaaaaagagc catgacacat ttataaaatg tttgacgaca 3300
ttttataaat gcatagcccg ataagattgc caaaccaacg cttatcagtt agtcagatga 3360
actcttccct cgtaagaagt tatttaatta actttgtttg aagacggtat ataaccgtac 3420
tatcattata tagggaaatc agagagtttt caagtatcta agctactgaa tttaagaatt 3480
gttaagcaat caatcggaaa tcgtttgatt gctttttttg tattcattta tagaaggtgg 3540
agtttgtatg aatcatgatg aatgtaaaac ttatataaaa aatagtttat tggagataag 3600
aaaattagca aatatctata cactagaaac gtttaagaaa gagttagaaa agagaaatat 3660
ctacttagaa acaaaatcag ataagtattt ttcttcggag ggggaagatt atatatataa 3720
gttaatagaa aataacaaaa taatttattc gattagtgga aaaaaattga cttataaagg 3780
aaaaaaatct ttttcaaaac atgcaatatt gaaacagttg aatgaaaaag caaaccaagt 3840
taattaaaca acctatttta taggatttat aggaaaggag aacagctgaa tgaatatccc 3900
ttttgttgta gaaactgtgc ttcatgacgg cttgttaaag tacaaattta aaaatagtaa 3960
aattcgctca atcactacca agccaggtaa aagcaaaggg gctatttttg cgtatcgctc 4020
aaaatcaagc atgattggcg gtcgtggtgt tgttctgact tccgaggaag cgattcaaga 4080
aaatcaagat acatttacac attggacacc caacgtttat cgttatggaa cgtatgcaga 4140
cgaaaaccgt tcatacacga aaggacattc tgaaaacaat ttaagacaaa tcaatacctt 4200
ctttattgat tttgatattc acacggcaaa agaaactatt tcagcaagcg atattttaac 4260
aaccgctatt gatttaggtt ttatgcctac tatgattatc aaatctgata aaggttatca 4320
agcatatttt gttttagaaa cgccagtcta tgtgacttca aaatcagaat ttaaatctgt 4380
caaagcagcc aaaataattt cgcaaaatat ccgagaatat tttggaaagt ctttgccagt 4440
tgatctaacg tgtaatcatt ttggtattgc tcgcatacca agaacggaca atgtagaatt 4500
ttttgatcct aattaccgtt attctttcaa agaatggcaa gattggtctt tcaaacaaac 4560
agataataag ggctttactc gttcaagtct aacggtttta agcggtacag aaggcaaaaa 4620
acaagtagat gaaccctggt ttaatctctt attgcacgaa acgaaatttt caggagaaaa 4680
gggtttaata gggcgtaata acgtcatgtt taccctctct ttagcctact ttagttcagg 4740
ctattcaatc gaaacgtgcg aatataatat gtttgagttt aataatcgat tagatcaacc 4800
cttagaagaa aaagaagtaa tcaaaattgt tagaagtgcc tattcagaaa actatcaagg 4860

ggctaatagg gaatacatta ccattctttg caaagcttgg gtatcaagtg atttaaccag 4920
taaagattta tttgtccgtc aagggtggtt taaattcaag aaaaaaagaa gcgaacgtca 4980
acgtgttcat ttgtcagaat ggaaagaaga tttaatggct tatattagcg aaaaaagcga 5040
tgtatacaag ccttatttag tgacgaccaa aaaagagatt agagaagtgc taggcattcc 5100
tgaacggaca ttagataaat tgctgaaggt actgaaggcg aatcaggaaa ttttctttaa 5160
gattaaacca ggaagaaatg gtggcattca acttgctagt gttaaatcat tgttgctatc 5220
gatcattaaa gtaaaaaaag aagaaaaaga aagctatata aaggcgctga caaattcttt 5280
tgacttagag catacattca ttcaagagac tttaaacaag ctagcagaac gccctaaaac 5340
ggacacacaa ctcgatttgt ttagctatga tacaggctga aaataaaacc cgcactatgc 5400
cattacattt atatctatga tacgtgtttg ttttttcttt gctgtttagc gaatgattag 5460
cagaaatata cagagtaaga ttttaattaa ttattagggg gagaaggaga gagtagcccg 5520
aaaactttta gttggcttgg actgaacgaa gtgagggaaa ggctactaaa acgtcgaggg 5580
gcagtgagag cgaagcgaac acttgatttt ttaattttct atcttttata ggtcattaga 5640
gtatacttat ttgtcctata aactatttag cagcataata gatttattga ataggtcatt 5700
taagttgagc atattagagg aggaaaatct tggagaaata tttgaagaac ccgattacat 5760
ggattggatt agttcttgtg gttacgtggt ttttaactaa aagtagtgaa tttttgattt 5820
ttggtgtgtg tgtcttgttg ttagtatttg ctagtcaaag tgattaaata 5870

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07K 14/54, 14/715

A3

(11) International Publication Number: **WO 00/23471**

(43) International Publication Date: 27 April 2000 (27.04.00)

(21) International Application Number: PCT/EP99/07800

(22) International Filing Date: 6 October 1999 (06.10.99)

(30) Priority Data:
98203529.7 20 October 1998 (20.10.98) EP

(71) Applicant *(for all designated States except US)*: VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW [BE/BE]; Rijvisschestraat 120, B–9052 Zwijnaarde (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: STEIDLER, Lothar [BE/BE]; Bokslaarstraat 41, B–9160 Lokeren (BE). REMAUT, Erik, Rene [BE/BE]; Bergstraat 7, B–9921 Vinderhoute (BE). FIERS, Walter [BE/BE]; Beukendreef 3, B–9070 Destelbergen (BE).

(74) Common Representative: VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW; Rijvisschestraat 120, B–9052 Zwijnaarde (BE).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
3 August 2000 (03.08.00)

(54) Title: USE OF A CYTOKINE–PRODUCING *LACTOCOCCUS* STRAIN TO TREAT COLITIS

(57) Abstract

The current invention relates to an administraton strategy for the delivery at the intestinal mucosa of cytokines or cytokine antagonists, preferably of acid sensitive anti–inflammatory agents, such as IL10 and/or soluble TNF receptor via the oral route. The prefered feature according to the invention is the inoculation with a suspension of recombinant *Lactococcus lactis* cells, which had been engineered to produce the respective proteins.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/EP 99/07800

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/54 C07K14/715

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 11277 A (DOMPE SPA ;TAGLIABUE ALDO (IT); BORASCHI DIANA (IT); BOSSU PAOLA () 18 April 1996 (1996-04-18) page 2, line 6 - line 11; claims 1-6,8 page 4, line 22 - line 27 page 6, line 29 -page 7, line 6 page 10, line 2 -page 11, line 20 page 11, line 28 -page 12, line 2 page 13, line 25 -page 15, line 2	1-6
A	WO 97 14806 A (UNIV CAMBRIDGE TECH ;STEIDLER LOTHAR (BE); REMAUT ERIK (BE); WELLS) 24 April 1997 (1997-04-24) cited in the application page 8, line 22 -page 9, line 6; claims 1,7,8,12,18 page 11, line 19 - line 25 page 12, line 23 -page 13, line 27	1-5

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 April 2000	02/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Charles, D

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. Jonal Application No
PCT/EP 99/07800

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	STEIDLER L ET AL: "Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant strains of Lactococcus lactis coexpressing antigen and cytokine." INFECTION AND IMMUNITY, (1998 JUL) 66 (7) 3183-9. JOURNAL CODE: G07. ISSN: 0019-9567., XP002105819 United States the whole document	1,3,6

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Jonal Application No
PCT/EP 99/07800

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9611277 A	18-04-1996	IT 1270123 B	28-04-1997
		AU 3745395 A	02-05-1996
		CA 2201721 A	18-04-1996
		EP 0784689 A	23-07-1997
		JP 10506791 T	07-07-1998
WO 9714806 A	24-04-1997	AU 7315496 A	07-05-1997
		BR 9610929 A	21-12-1999
		CN 1202934 A	23-12-1998
		EP 0871748 A	21-10-1998
		NO 981746 A	22-06-1998